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Item 3 of the provisional agenda

CONSIDERATION OF PROJECT PROPOSALS BY GOVERNMENTS AND INDUSTRY WHICH COULD BE SUBMITTED FOR POSSIBLE FINANCING UNDER THE SECOND ACCOUNT OF THE COMMON FUND FOR COMMODITIES

Project proposals received by the UNCTAD secretariat

PROPOSALS BY THE INTERNATIONAL TUNGSTEN INDUSTRY ASSOCIATION

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Note by the UNCTAD secretariat

The Secretary-General of UNCTAD has received from the International Tungsten Industry Association (ITIA) a communication dated 20 January 1992 transmitting the project proposal reproduced below.

The communication included the following comments:

"In accordance with the requirements laid down at the last meeting of the UNCTAD Committee on Tungsten, the ITIA, as a Committee member, hereby encloses a project which it hopes the Committee will sponsor for submission to the Common Fund, for funding under its second account. The project reflects the tungsten industry's commitment to the health and safety of people in the workplace and to the environment worldwide.

"We look forward to receiving the comments of other Committee members and to learning the date of the mid-year ad hoc meeting so that expert representation can be arranged to supply any additional technical explanations which the Committee may require."

* * *

PROJECT PROPOSAL FOR FINANCING THROUGH THE SECOND ACCOUNT

The UNCTAD Committee on Tungsten hereby submits the following project proposal with its recommendation for financing through the Second Account of the Common Fund.

Project title:	HEALTH AND ENVIRONMENTAL EFFECTS TESTING OF TUNGSTEN MATERIALS
Duration:	The project is estimated to require 40 weeks to complete.
Location:	The project will be conducted at Huntingdon Research Centre Ltd., Huntingdon, United Kingdom. The studies will be conducted in accordance with internationally accepted protocols.
Nature:	Health and environmental with associated benefits for worker safety, environmental protection and the potential for competitive advantage.
Total cost:	£200,000
Financing sought from the Common Fund:	£200,000
Co-financing sought:	£0
Co-financing committed:	£0
Counterpart contribution:	£0
Project Executing Agency:	Huntingdon Research Centre Ltd., Huntingdon, United Kingdom.
Estimated starting date:	As soon as possible.

A. PROJECT DESCRIPTION

(a) Protection of the health of workers, the general public, and the environment from the possible adverse effects of industrial chemicals and products has been and continues to be a global concern. Among other activities, protection of health and the environment have led to government regulation of environmental hazards and the need to develop environmentally safe substitutes for those materials known to be harmful to human health and/or the environment.

Tungsten itself is generally considered to be of low toxicity. Although the relationship between lung disease ("hard metals disease") and exposure to certain grades of tungsten carbide, namely those containing cobalt, has been extensively studied, the literature contains relatively little data on the toxic and environmental effects of pure tungsten materials. The development

of health and environmental effects data for pure tungsten materials, using internationally accepted protocols, will promote the use of tungsten in applications where tungsten may be substituted for environmentally hazardous materials; i.e., lead, cadmium, chromium, if the study results show tungsten to be of low toxicity. Also, since Governments tend to take a conservative approach to chemical regulations where little definitive evidence exists that a material does not present a hazard, the proposed studies will help assure that any future environmental regulations pertaining to tungsten materials are based on sound data.

(b) The overall objective of the project will be to develop a comprehensive set of health and environmental data on tungsten materials of commercial importance. The strategy will be to use internationally accepted testing protocols to develop key indicators of short-term or acute health and environmental effects, and also to investigate the subchronic inhalation effects of pure tungsten materials as compared to tungsten-carbide containing cobalt.

The project will benefit tungsten producers, users and workers. If the study results show low toxicity, tungsten will have a competitive advantage over competing materials with greater potential hazard. The study results will allow such comparisons to be made based on objective data. If any adverse effects are found as a result of the study, tungsten users and producers will be able to minimize those effects through protective devices, warnings, etc. Tungsten industries and government agencies charged with worker safety, public health, and environmental protection will benefit since the data generated by the project will enable these agencies to make informed regulatory decisions. In a broad sense, society in general will benefit for these same reasons.

At the end of the project it is expected that key toxicity and environmental data (described below) will exist and be distributed to interested parties in government and industry.

The International Tungsten Industry Association has had extensive discussions with Huntingdon Research Centre Ltd., Huntingdon, Cambridgeshire, United Kingdom. HRC, which is a leading independent toxicological research laboratory, has provided the International Tungsten Industry Association with a suggested testing programme and detailed testing protocols and costs.

B. PROJECT JUSTIFICATION

(a) The benefits associated with this project, such as increased knowledge, potentially increased competitiveness, and cost-effective regulation, are intangible and impossible to quantify in financial terms.

(b) The project will be conducted using internationally recognized testing protocols including Good Laboratory Practices set forth by the United States Environmental Protection Agency, the Organisation for Economic Co-operation and Development, the European Community, the Japanese Ministry of International Trade and Industry and the United Kingdom Department of Health. No technical difficulties in conducting the project are anticipated.

(c) The purpose of this project is to provide a profile of health and environmental data. The project is therefore expected to have positive environmental implications.

(d) The results of this study are expected to provide industries and Governments in developed and developing countries with data upon which to make sound and cost-effective decisions regarding worker safety and environmental controls. This can lead to a positive socio-economic impact by promoting a safe work environment without overly burdensome regulation.

(e) This project is consistent with the objectives and priorities of the Common Fund. The project has a great potential to benefit the structural developments of the tungsten industry. Several UNCTAD studies have recently shown how the tungsten industry has suffered from technological changes, e.g., the increased use of other materials such as cermets, ceramics, coatings, and near-net-shape techniques. The information and knowledge gained from this project will be valuable, in accordance with the terms and provisions of the Common Fund, in promoting the use of tungsten materials against competitive materials as research reveals new applications. The project will benefit both developed and developing producer and user countries. Likewise, the project is expected to have positive environmental and socio-economic impacts.

C. OBJECTIVES

Objective 1: To develop acute toxicity data on four tungsten materials: tungsten metal, tungsten carbide, ammonium paratungstate, and sodium tungstate.

Outputs: Acute lethal oral dose (LD50) to rats and associated clinical signs and pathology.

Acute lethal inhalation dose (LC50) to rats and associated clinical signs and pathology.

Acute lethal dermal dose to rats and associated clinical signs and pathology.

Irritant effects on rabbit eye.

Irritant effects on rabbit skin.

An assessment of the potential for tungsten materials to cause delayed contact hypersensitivity.

Details of the testing protocols can be found in Annex I.

Objective 2: To develop acute ecotoxicity data on four tungsten materials: tungsten metal, tungsten carbide, ammonium paratungstate, and sodium tungstate.

Outputs: Acute lethal concentration (LC50) to rainbow trout.

Acute toxicity to Daphnia (24-hour and 48-hour immobilization values).

The extent of inhibition of micro-organisms at various concentrations of test material.

The extent of algae growth inhibition at various concentrations of test materials (EC50).

Details of the testing protocols can be found in Annex II.

Objective 3: To assess the repeated dose inhalation toxicity of tungsten and tungsten carbide in rats.

Outputs: Mortality and morbidity, clinical signs, haematologic and biochemical measurements, and histopathology of organs, with emphasis on the lungs and tracheo-bronchial lymph nodes. Use of a tungsten carbide-cobalt mixture as a comparative control in this study will allow a comparison of the effects on rats of pure tungsten and pure tungsten carbide with the effects of tungsten carbide/cobalt mixture, a substance associated with "hard metals disease" in man.

Details of the testing protocol can be found in Annex III.

D. INPUTS

The project would be conducted by an independent contract research laboratory (the Project Executing Agency). All equipment, personnel, and supplies necessary to carry out the project, as outlined in the testing protocols (annexes I, II and III), would be provided by the laboratory as part of the contract.

Samples of tungsten materials to be tested will be supplied by member companies of the International Tungsten Industry Association.

The services of an independent testing laboratory would be needed to assay the samples of tungsten materials to be used as the test substances by the Project Executing Agency.

E. RISKS

This project will be conducted using standard equipment and procedures. No significant risks are anticipated. Delays in the start-up of individual tests, particularly the repeated inhalation study, may occur due to exposure chamber scheduling requirements. Such delays however will not interfere with the achievement of the project's outputs and objectives.

F. PROJECT BUDGET

(a) Project income. The International Tungsten Industry Association (the project's conceiver) has contributed considerably in terms of time, research, and travel by some of its members and staff. The UNCTAD Committee on Tungsten will contribute to the project by serving as the Supervisory Body, which may likely call for the hiring of an expert consultant to oversee the project which will need to be supervised on a regular basis, from both technical and administrative viewpoints. Monies for conducting the studies are being requested from the Common Fund as outlined below.

(b) Project cost. The following costs are being sought from the Common Fund. Note that the main costs associated with this project are for contracts and services to be performed by an independent toxicology laboratory. Personnel, administration, and equipment will be provided by the laboratory and included in the testing costs.

Contracts and services	£	£
Acute oral toxicity	4 360	
Acute dermal toxicity	4 740	
Acute inhalation toxicity	26 000	
Skin irritation	1 120	
Eye irritation	1 120	
Skin sensitization	6 200	
Acute trout toxicity	5 436	
Acute Daphnia toxicity	4 716	
Bacterial inhibition	4 176	
Algae growth inhibition	5 184	
Repeated dose inhalation	82 850	
Analytical services	2 500	
Total contracts and services		148 402
Consultancy fee		30 000
Official travel		10 000
Contingency		11 598
Total funds requested from Common Fund		200 000

As mentioned in Section B, paragraph (b), these tests follow standard procedures and programmes set by many government regulatory authorities.

G. PROVISIONAL WORK PLAN

Most of the testing will be conducted simultaneously in various departments of Huntingdon Research Centre Ltd. Each test is independent of all the others. The acute toxicity testing is expected to be completed within 16 weeks of the start of the project; acute ecotoxicity testing within 8 weeks, and the repeated dose inhalation study within 40 weeks of the start of the project.

H. PROJECT MANAGEMENT

It is proposed that Huntingdon Research Centre Ltd. (HRC), Huntingdon, United Kingdom, serve as the Project Executing Agency. Copies of a brochure describing the services offered by HRC are available on request.

The UNCTAD Committee on Tungsten will assume the responsibility of project Supervisory Body. If deemed necessary, the Committee on Tungsten will hire expert consultants and/or any organization within the United Nations which could contribute to the conduct of the project.

I. PROJECT MONITORING, REPORTING AND EVALUATION

The Committee on Tungsten (the supervisory body) will monitor and evaluate the project progress, directly or through an expert consultant, by liaison with HRC. It is expected that HRC (the project executing agency) will provide a report on the status of each test every three months. At the completion of the study, HRC will provide the Committee on Tungsten with a detailed report on each test, describing the test procedures and results. The Committee on Tungsten will forward all reports from HRC to the Fund.

ANNEXES

I Protocols for acute toxicity tests.
II Protocols for acute ecotoxicity tests.
III Protocols for repeated dose inhalation test.

On Behalf of The UNCTAD Committee on Tungsten

Name	Signature
Title	Date

ANNEX I

PROTOCOL

ACUTE ORAL TOXICITY TO THE RAT

1. Introduction

Study objective:	Assessment of toxicity following a single oral dose.
Protocol:	In compliance with: EEC Methods for the determination of toxicity. Directive 84/449/EEC (OJ No. L251, 19 September 1984), Part B, Method B1 Acute toxicity (oral).
Test species:	Albino rat.
Justification for selection of species:	Experience with this species and compliance with test guideline.
Route of administration:	Oral gavage.
Justification for route of administration:	Possible route of human exposure.
Duration of study:	Fourteen days (or until signs of reversible toxicity subside, if this is beyond 14 days).

2. Test substance

2.1. The test substance, when possible, will be administered as supplied, at a variable dose volume.

2.2. Dilutions of liquid test substances may be made where the toxicity of the test substance is high and the dose volume would be too low to ensure accurate administration of the undiluted sample.

2.3. Viscous liquids and solids are suspended or dissolved in suitable non-toxic vehicles. Suitable vehicles for use are distilled water, physiological saline, 1 per cent w/v aqueous methylcellulose or corn oil. The concentration of the test substance is varied so as to permit constant volume dosing. The volume administered at one time will not exceed 20 ml/kg for aqueous, or 10 ml/kg for non-aqueous solutions/suspensions.

2.4. If the physical characteristics of the test substance require administration of dose volumes above the limits given in 2.3., the dose may be given in smaller fractions over a period not exceeding 24 hours.

2.5. The test substance, when formulated, will be prepared on the day of dosing.

2.6. The test substance will be stored at room temperature in non-continuous artificial light unless otherwise requested by the Sponsor on the Test Substance Data Sheet.

2.7. The absorption of the test substance will not be determined in this study.

2.8. Data concerning the stability, homogeneity and purity of the test substance is the responsibility of the Sponsor.

2.9. Unless the Sponsor indicates that the test substance is physically or chemically unstable in the proposed vehicles, assays of dose formulations will not be undertaken at HRC.

3. Animal management

3.1. Equal numbers of healthy male and female rats of Sprague-Dawley origin will be obtained from a commercial breeder. Animals will be in the weight and age ranges 70 to 150 g and four to seven weeks of age prior to dosing on Day 1. The weight variation within each sex will not exceed ± 20 per cent of the mean weight. All the rats will be acclimatized to the experimental environment for a minimum of five days prior to allocation to the test.

3.2. The animals will be allocated to groups and cages without conscious bias. Up to five animals of the same sex will be housed in metal cages with wire mesh floors.

3.3. Animals will have free access to tap water and Biosure LAD 1 rodent diet or suitable alternative. Access to food will be prevented overhight prior to dosing and for approximately four hours after dosing.

3.4. Analyses will be made on all batches of the diet used to establish levels of nutrients, possible contaminants and micro-organisms likely to be present in the diet, and which, if in excess, may have an undesirable effect on the test system. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.5. Results of routine physical and chemical examination of drinking water at source (Grafham Final Water - Huntingdon North supply zone) as conducted usually weekly by the supplier, Anglian Water Services Ltd., are made available to Huntingdon Research Centre as quarterly summaries. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.6. Animal room temperature and relative humidity will be recorded daily. Thermostatic controls will be set to achieve a temperature of 22 ± 3° C. Relative humidity is not controlled but is anticipated to be in the range 30-70 per cent R.H.

3.7. Natural lighting will be supplemented with 12 hours of artificial light (0700-1900 hours) in each 24-hour period.

3.8. Each animal at each dose level will be identified by cage number and ear punching or tail tattooing.

3.9. Each cage will be identified by a coloured label. Each label will display the dose level, study schedule number, animal mark and the initials of the Study Director and Home Office licensee.

4. Treatment procedure

4.1. Preliminary study

If toxicity is suspected at or below 5.0 g/kg bodyweight, preliminary testing will be carried out to establish a dosing regimen for the main study using groups of two male and two female rats at a range of dose levels which generally will include at least one dose level below and one dose level above the expected LD_{50}.

4.2. Limit test

If toxicity is not suspected a group of five male and five female rats will be treated at 5.0 g/kg bodyweight.

4.3. Main study

4.3.1. When mortality occurs at 5.0 g/kg bodyweight or below, the initial dose level(s) will be selected on the basis of the preliminary study (Section 4.1.). An additional level(s) may be chosen after review of the results. At least three dose levels will be used, each consisting of five male and five female rats. Doses will be chosen to include at least one dose level below and one dose level above the expected LD_{50}.

4.3.2. Prior to dose administration the Study Director will authorize the choice of dose levels in the raw data.

4.4. Control animals

No control animals will be included in this study.

4.5. Administration of test substance

4.5.1. All animals will be dosed by oral gavage via a plastic syringe and catheter. Glass syringes, metal cannulae or rubber catheters may be used if the test substance is suspected of incompatibility with plastic. If the test substance is prepared in a vehicle or diluent, the concentrations will be adjusted to achieve the necessary levels.

4.5.2. Treatment in this manner will be performed on Day 1 of the study only.

5. Observations

5.1. Mortality

5.1.1. Where deaths occur, the approximate time of death of individual rats will be recorded and animals examined as described in 6.2. and 6.3. below.

5.1.2. Where necessary, animals showing severe signs of toxicity will be isolated.

5.1.3. Animals showing severe reactions which are likely to persist and moribund animals will be killed.

5.2. Clinical signs

5.2.1. Animals will be observed soon after dosing and then at frequent intervals for the remainder of Day 1. On subsequent days the animals will be observed once in the morning and once at the end of the experimental day (this latter observation will be at approximately 1130 hours on Saturday and Sunday). The nature, severity, approximate time of onset and duration of each sign of toxicity will be recorded at each observation.

5.2.2. The animals in the preliminary study (Section 4.1.) will be observed for a minimum of five days. The animals in the limit test (Section 4.2.) or the main study (Section 4.3.) will be observed for at least 14 days after dosing or until all signs of reversible toxicity subside, if this is beyond 14 days.

5.3. Bodyweight

The bodyweight of each rat in the limit test (Section 4.2.) or the main study (Section 4.3.) will be recorded on Days 1 (prior to dosing), 8 and 15 or at death. Where possible, group mean bodyweight data will be calculated.

6. Post-mortem examination

6.1. Surviving animals in the main study (Section 4.3.) or animals in the limit test (Section 4.2.) will be killed on Day 15 (unless delayed toxicity is observed and extension of the study is considered necessary to achieve the study objective).

6.2. All animals that die or are killed will be subjected to a macroscopic post-mortem examination which will consist of opening the thoracic and abdominal cavities. The cranial cavity will also be examined if observations indicate neurotoxic activity. The macroscopic appearance of abnormal organs, when present, will be recorded.

6.3. Abnormal organs/tissues of animals surviving more than 24 hours may be preserved in suitable fixative pending any future requirement for microscopic pathological examination.

7. Statistical analysis

The LD_{50} value, and where possible, the value of males and females separately, will be calculated from the observed mortality data, using established procedures.

References:

Finney, D.J., (1971) Probit Analysis, 3rd edn., Cambridge University Press;

Weil, C.S. (1952) Biometrics, 8, 249;

Litchfield, J.T., and Wilcoxon, F., (1949) J. Pharmac. Exp. Ther. 96, 99.

8. Reports

Unless otherwise requested, six bound copies of the report will be issued. The report will include the following:

- A description of the test substance;
- The species/strain of the test animal;
- Full details of the experimental procedures used;
- A summary of observations on limit test and/or main study, including tabulation of signs of reaction to treatment by sex and dose level and time of deaths;
- Bodyweight data;
- The calculated LD_{50} value, with associated 95 per cent confidence limits, for all animals and, where possible, for males and females separately;
- Dose mortality curve(s), where appropriate;
- Autopsy findings.

9. Good laboratory practice

9.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

United States Environmental Protection Agency, (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Organisation for Economic Co-operation and Development
ISBN 92-64-12367-9, Paris 1982.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

9.2. Amendments to protocol

The Study Director will normally seek the approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3. Quality Assurance Department review

The Quality Assurance Department will:

(a) Conduct inspections of various phases of the study itself and/or of certain repetitive procedures relevant to the study design, and

(b) Audit the final report.

9.4. Maintenance of records

Following dispatch of the final report, all raw data and other documents generated by HRC during the course of this study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

PROTOCOL

ACUTE INHALATION TOXICOLOGY STUDY IN RATS

LIMIT TEST OR

DETERMINATION OF THE LD_{50} (4 HOUR)

WHOLE BODY EXPOSURE*

INTRODUCTION

The study described in this protocol is designed to investigate the acute inhalation toxicity of the test substance and, if appropriate, to determine the median lethal concentration LC_{50} (4 hour). The study is designed to be in compliance with all current test guidelines (OECD, EEC, US-EPA, Japanese MAFF) for this type of study, although, in certain instances, the guideline requirement may be exceeded. The procedures to be used during the course of this study are those documented in the Department of Inhalation Toxicology Standard Operating Procedures Manual.

1. Purpose

The object of this study is to assess the response of rats to inhalation exposure, for a single period of four hours, to a test atmosphere containing the test substance.

Initially a group of rats will be exposed at a concentration of 5 mg of test substance per litre of air or to the maximum attainable concentration, whichever is the lower. If mortality exceeds 50 per cent further groups of rats will be exposed in order to provide sufficient data for estimation of the median lethal concentration (LC_{50}) for the test substance.

2. Test animals

2.1. Species and strain: Virgin young adult rats of Sprague Dawley origin.

2.2. Supplier: The rats will be ordered from Charles River Ltd. The rats may be supplied from Charles River (United Kingdom), Charles River (France) or Charles River (United States of America) dependent on the health status of the colony.

2.3. Number: Possible total of 60 (30 male and 30 female). One control group and one to five test groups each of five male and five female rats

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* Full and exact details of the conduct of this study cannot be given at this time. Further information will be sent after HRC is satisfied that the study can be performed within the general outline given in this protocol. This information will be sent on a study details form, a copy of which is appended to this protocol. However, please indicate your acceptance of the outline for the study by signing and dating one of the attached protocol approval pages and returning it to us as soon as possible.

2.4. Age: Six to eight weeks.

2.5. Weight: The rats will be ordered by weight so as to weigh approximately 200g on the day of exposure.

2.6. Justification of choice of species: The rat is a species recommended by all current test guidelines for this type of study. In addition, HRC has had many years experience in the use of the rat as an animal model in toxicology studies, and comprehensive background data.

2.7. Selection of animals: Any rat that is in poor condition or physically damaged will not be allocated to the study.

2.8. Allocation: If the test substance is of low toxicity or if there is no data available to evaluate likely toxicity only 20 (10 male and 10 female) rats will be ordered initially. These will be randomly allocated to form two groups:

Group 1 (Control) - five male + five female

Group 2 (Test) - five male + five female.

If further groups are to be exposed, these will be ordered as required and randomly allocated to groups where the delivery consists of more than one group.

Where the substance is considered likely to be toxic up to 60 rats (30 male and 30 female) may be obtained in one delivery and allocated to groups on arrival.

2.9. Identification: Each rat will be uniquely identified by a number tattooed on the ear pinnae.

2.10. Acclimatization: The rats will be held in the HRC laboratories for at least five days prior to inhalation exposure.

2.11. Dead and moribund animals: Any rat that dies during the acclimatization period will be replaced by a rat of the same sex and similar bodyweight.

Should any rat die or be sacrificed for humane reasons at any other time during the study it will be subjected to a complete macroscopic examination.

The lungs, liver, kidneys and macroscopic abnormalities will be preserved (see sections 7.1. and 7.2.) if microscopic pathological examination has been requested.

3. Accommodation and Husbandry

3.1. Location of study: Building M12.

3.2. Cage type and size: Stainless steel mesh on front, back and floor with stainless steel sheet sides: 35 cm wide, 53 cm long and 25 cm high. The cages are suspended on racks. Plastic trays, lined with absorbent paper, will be placed below the cages to collect animal waste.

3.3. Number of animals per cage: Five of like sex.

3.4. Cage identification: Each cage will bear a coloured label identifying the study number, the treatment group, the sex and numbers of the rats within the cage.

3.5. Room temperature: The room thermostat will be set at 21° C.

The temperature will be recorded continuously using a Kent Clearspan recorder.

3.6. Room relative humidity: Relative humidity will be recorded continuously using a Kent Clearspan recorder.

3.7 Lighting: 12 hours light (0800-2000) and 12 hours dark.

3.8. Dry diet: SDS rat and mouse diet will be available ad libitum except during exposure.

3.9. Water: Tap water from moulded polypropylene bottles will be available ad libitum except during exposure. The bottles will be rinsed and refilled daily.

3.10. Analysis of food and water: There is no information available to indicate that any substance likely to influence the effect of the test compound can reasonably be expected to be present in the diet or drinking-water.

Analysis will be made of all batches of diet for most nutrients and for specified substances and micro-organisms likely to be present in feed ingredients or the finished diet and which, if in excess of specified amounts, might have an undesirable effect on the test system. Although occasional slight deviation may be permitted, batches of diet will conform with the acceptable standards agreed by the Study Director and Head, Quality Assurance Department.

The analytical data will be lodged in HRC Archives.

The water supplied to HRC, by Anglian Water, is potable water for human consumption. Anglian Water takes its guidelines on water quality from the EEC directive relating to water for human consumption, viz: Council Directive 80/778/EEC.

Results of routine physical and chemical examination of drinking-water at source as conducted, usually weekly by the supplier, are made available to HRC as quarterly summaries.

These results include levels of:

Nitrites	Potassium	Chloride
Nitrates	Silicon	Iron
Calcium	Arsenic	Selenium
Magnesium	Barium	Silver
Sodium	Antimony	Phosphorus

as well as concentrations of pesticides, related products, polycyclic aromatic hydrocarbons, haloforms, chlorophenols and polychlorinated biphenyls.

4. Inhalation Exposure

4.1. Duration: A single four-hour continuous exposure. The exposure will be timed for four hours following an equilibration period during which the concentration of the test substance is expected to attain 90 per cent of its final concentration.

4.2. Exposure chambers: Exposure will take place in perspex chambers of approximately 120 litres capacity. The chambers are divided internally by metal mesh partitions which separate the rats during exposure.

The chambers are of square section and are fitted with pyramidal tops. The test atmosphere normally enters from the base centre or top centre of the chamber, passes through the chamber and exits through a series of holes distributed evenly round the base of the chamber below the level of the animals.

The chambers will be operated under dynamic airflow conditions. The flow of air to each chamber will be within the range 20-30 litres/minute but will be constant throughout. The rate of airflow will be monitored throughout exposure and will be recorded at 30 minute intervals.

4.3. Test atmosphere temperature and relative humidity: The temperature in the exposure chamber will be monitored continuously during exposure and recorded at 30 minute intervals.

If possible the chamber humidity will be monitored continuously using an infrared water vapour analyser or a Casella type T6900 relative humidity meter and recorded at 30 minute intervals. This measurement is not normally possible where the test substance is present as droplets or vapour.

4.4. Generation of the test atmosphere:

A stainless steel or glass concentric jet atomizer is normally used to produce test atmospheres from liquid test substances.

A Wright dust-feed mechanism or a turntable dust-feed mechanism is normally used to produce test atmospheres from particulate test substances.

In the event that liquid or particulate test substances are not amenable to aerosolization by the above techniques, the test aerosol may be produced by atomization of a solution of the test substance in an appropriate low toxicity solvent. This technique would be employed only after the Sponsor's approval.

An all-glass evaporation system is normally used to produce test atmospheres containing vapour of the test substance where the test substance has a high vapour pressure at room temperature.

Alternative equipment is available for test substances that cannot be readily dispersed in air using any of the above methods.

4.5. Atmosphere analysis: Concentration - At least five samples of the chamber air will be removed in order to estimate the concentration of the test substance in the chamber air. For liquid droplet aerosol or vapour-containing atmospheres, collection will normally be by a sintered glass bubbler containing a suitable solvent. For particulate and low volatility droplet aerosols, collection will be by a glass fibre filter held in an open-face filter holder.

Sample volumes will be measured using a wet-type gas meter.

A gravimetric and/or chemical determination of the amount of test substance collected on the filters will be performed. Samples collected using bubblers will be analysed chemically. Chemical analysis of formulations will be for a single selected active ingredient. Additional components may be analysed at extra cost.

Particle size distribution - At least two samples will be taken during the four-hour exposure for determination of particle size distribution.

For particulate and low volatility droplet aerosols, the size distribution by weight of the particle cloud will be estimated using a Marple model 296 cascade impactor. Alternative methods may be used depending on the nature of the test material.

If the test substance is all present as vapour at the concentration tested, particle size analysis will not be applicable.

4.6. Control group: A similar exposure system will be used for the control group. Sections 4.2. and 4.3. will apply except that the rats will be exposed to clean air only. (If a solvent has been used to aid aerosolization of the test substance, the control group will be exposed to the solvent only). The duration of exposure will be four hours plus the equilibration time.

5. Procedure

5.1. Preliminary experiments: Preliminary experiments will be undertaken to establish the suitability of the test atmosphere generation system for use with the test substance. Particular attention will be given to stability of the concentration of the test substance.

At this stage exposure of a small number of rats may be undertaken, particularly if it is suspected that the test substance is likely to be highly toxic at 5 mg/l or the highest level attainable.

5.2. Exposure: A group of five male and five female rats will be exposed at 5 mg/l or the maximum attainable concentration, whichever is the lower unless high toxicity has been shown during preliminary work. In the latter case the exposure will be at a level expected to be consistent with the survival of some rats.

If exposure at 5 mg/l results in a mortality in excess of 50 per cent during the study period further groups of five male and five female rats will be exposed at lower concentrations until we are able to assess the LC_{50}. A maximum of four additional groups of rats will be used.

If there is a clear difference in the response of male and female rats, groups of rats of one sex only may be exposed in order to provide data to allow calculation of the LC_{50} for the sexes separately.

The control group will be subject to the same restraint and exposure procedures except that no test substance will be introduced into the exposure chamber.

6. Observations

6.1. Clinical signs: All rats will be observed at hourly intervals or more frequently during exposure. Signs of reaction to exposure will be recorded in terms of time of onset, duration and intensity (where appropriate). During the observation period a full clinical signs check will be performed daily in the morning. The rats will be checked for survival later in the day.

6.2. Deaths: The circumstances of any deaths will be recorded in detail, a full post-mortem examination carried out and the macroscopic condition of all major internal organs noted. The lungs will be removed, dissected free of surrounding tissue, and weighed.

The lung weight to bodyweight ratio will be calculated. The lungs, liver, kidneys and all macroscopically abnormal tissues will be preserved (see 7.2) if microscopic examination has been requested.

6.3. Bodyweight: All rats will be weighed daily from the day of their arrival at our laboratories up to and including the day of death.

6.4. Food consumption: The amount of food eaten by each cage of rats will be measured daily from the day following the arrival of the rats up to and including the day of death.

6.5. Water consumption: Individual water bottles attached to each of the cages will be weighed when filled and reweighed the following day in order to estimate the water intake by each cage of rats.

These measurements will be performed daily from the day after the arrival of the animals up to and including the day of death.

6.6. Observation period: The rats will be kept for an observation period of 14 days following exposure. This period may be extended at the discretion of the Study Director, if there is evidence of development of late toxicity or unusually slow recovery from the effects of exposure.

7. Terminal Studies

7.1. Terminal kill: Rats surviving the observation period will be anaesthetized by intraperitoneal injection of pentobarbitone sodium and exsanguinated. A complete macroscopic examination of each rat will be performed and lung weights will be recorded.

7.2. Tissue preservation: If the Sponsor has requested microscopic examination of the lungs, liver and kidneys by not deleting this option on the protocol, the lungs, liver, kidneys and all macroscopically abnormal tissues will be preserved in buffered 10 per cent formalin.

If microscopic examination has not been requested no tissues will be preserved.

7.3. Microscopic pathology: Microscopic examination of the lungs, liver and kidneys will be undertaken if the Sponsor has indicated his approval by not deleting the section on the quotation.

Microscopic examination of organs showing gross pathology in rats surviving 24 hours or more might provide additional useful information.

8. Evaluation of results

Where possible the median lethal concentration (LC_{50}) will be calculated using appropriate statistical procedures.

9. Archives

All raw data and microscope slides (where appropriate) will be lodged in the Huntingdon Research Centre Archives. The data will be retained for at least 10 years.

10. Reports

Five bound copies of a final report describing the procedures followed and detailing the results obtained will be prepared.

Appendix

STUDY DETAILS

The Protocol (Reference No. INH) was issued and approved by HRC Management and approved by the Sponsor

Sponsor:

HRC Schedule No.:

Location of study: (a) Exposures:

(b) Animal holding room:

Test substance:

Name: Batch No.:

Identity: Purity:

Storage conditions: Stability:

Generation apparatus:

Atmosphere analyses:

(1) Concentration of test substance:

Number of samples: Analyte: Method:

(2) Size distribution:

Number of samples: Apparatus:

Microscopic examination:

Study Analyst:

APPROVAL OF STUDY DETAILS

Study Director: Date:

Sponsor: Date:

TIME PLAN

Arrival of test substance:

Start of preliminary work:

Arrival of animals:

Start of main study:

Proposed reporting date:

Circulation list: Sponsor (x2), G.C. Clark, C.J. Hardy, G.C. Jackson, Acute Lab., Q.A.D., W. Jorgeson, L. Cooke.

PROTOCOL

ACUTE DERMAL TOXICITY TO THE RAT

1. Introduction

Study objective:	Assessment of toxicity following a single dermal dose.
Protocol:	In compliance with: EEC Methods for the determination of toxicity, Directive 84/449/EEC (OJ No. L251, 19.9.84), Part B, Method B3 Acute toxicity (dermal).
Test species:	Albino rat.
Justification for selection of species:	Experience with this species and compliance with test guideline.
Route of administration:	Topical application.
Justification for route of administration:	Possible route of human exposure.
Duration of study:	Fourteen days (or until signs of reversible toxicity subside, if this is beyond 14 days).

2. Test substance

2.1. The test substance, when possible, will be administered as supplied, at a variable dose volume.

2.2. Dilutions of liquid test substances may be made where the toxicity of the test substance is high and the dose volume would be too low to ensure accurate administration of the undiluted sample or where the material is a severe irritant or is corrosive.

2.3. If the test substance is a solid it may be pulverised and will be moistened with water or other non-toxic vehicle to form a paste before application.

2.4. The test substance, when formulated, will be prepared on the day of dosing.

2.5. The test substance will be stored at room temperature in non-continuous artificial light unless otherwise requested by the Sponsor on the Test Substance Data Sheet.

2.6. The absorption of the test substance will not be determined in this study.

2.7. Date concerning the stability, homogeneity and purity of the test substance is the responsibility of the Sponsor.

2.8. Unless the Sponsor indicates that the test substance is physically or chemically unstable in the proposed vehicles, assay of dose formulations will not be undertaken at HRC.

3. Animal management

3.1. Equal numbers of healthy male and female rats of Sprague-Dawley origin will be obtained from a commercial breeder. Animals will be in the weight and age ranges 180-250 g and seven to 10 weeks of age prior to dosing on Day 1. The weight variation within each sex will not exceed ± 20 per cent of the mean weight. All rats will be acclimatised to the experimental environment for a minimum of five days prior to allocation to the test.

3.2. The animals will be allocated to groups and cages without conscious bias. The rats will be housed individually in metal cages with wire mesh floors.

3.3. Animals will have free access to tap water and Biosure LAD 1 rodent diet or suitable alternative.

3.4. Analyses will be made on all batches of the diet used to establish levels of nutrients, posible contaminants and micro-organisms, likely to be present in the diet, and which, if in excess, may have an undesirable effect on the test system. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.5. Results of routine physical and chemical examination of drinking water at source (Grafham Final Water - Huntingdom North supply zone) as conducted usually weekly by the supplier, Anglian Water Services Ltd., are made available to Huntingdon Research Centre as quaterly summaries. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.6. Animal room temperature and relative humidity will be recorded daily. Theromostatic controls will be set to achieve a temperature of 22 ± 3° C. Relative humidity is not controlled but is anticipated to be in the range 30-70 per cent RH.

3.7. Natural lighting will be supplemented with 12 hours of artificial light (0700-1900 hours) in each 24-hour period.

3.8. Each animal at each dose level will be identified by cage number and ear punching or tail tattooing.

3.9. Each cage will be identified by a coloured label. Each label will display the dose level, study schedule number, animal marks and the initials of the Study Director and Home Office licensee.

4. Treatment procedure

4.1. Preliminary study

If toxicity is suspected at or below 2.0 g/kg bodyweight, preliminary testing will be carried out to establish a dosing regimen for the main study using groups of two male and two female rats at a range of dose levels which generally will include at least one dose level below and one dose level above the expected LD_{50}.

4.2. Limit test

If toxicity is not suspected a group of five male and five female rats will be treated at 2.0 g/kg bodyweight.

4.3. Main study

4.3.1. When mortality occurs at 2.0 g/kg bodyweight or below, the initial dose level(s) will be selected on the basis of the preliminary study (Section 4.1.). An additional level(s) may be chosen after review of the results. At least three dose levels will be used, each consisting of five male and five female rats. Doses will be chosen to include at least one dose level below and one dose level above the expected LD_{50}.

4.3.2. Prior to dose administration the Study Director will authorize the choice of dose levels in the raw data.

4.4. Control animals

No control animals will be included in this study.

4.5. Administration of test substance

4.5.1. Twenty-four hours prior to treatment, hair will be clipped from the dorso-lumbar region of each rat exposing an area approximately 10 per cent of the total body surface area.

4.5.2. Unless the test substance is highly toxic it will be applied by spreading evenly over an area equivalent to approximately 5 cm x 5 cm of the clipped treatment site. With highly toxic substances, the surface area covered may be less, but as much of the area will be covered as possible. The treatment site will be covered with gauze backed with a non-irritating dressing, encircled firmly around the trunk of the animal.

4.5.3. Treatment in this manner will be performed on Day 1 of the study only.

4.5.4. After the exposure period of 24 hours, the dressing will be removed and the treatment site gently washed with water or appropriate solvent to remove residual test substance.

5. Observations

5.1. Mortality

5.1.1. Where deaths occur the approximate time of death of individual rats will be recorded and animals examined as described in 6.2. and 6.3. below.

5.1.2. Animals showing severe reactions which are likely to persist and moribund animals will be killed.

5.2. Clinical signs

5.2.1. Animals will be observed soon after dosing and then at frequent intervals for the remainder of Day 1. On subsequent days the animals will be observed once in the morning and once at the end of the experimental day (this latter observation will be at approximately 1130 hours on Saturday and Sunday). The nature, severity, approximate time of onset and duration of each sign of toxicity will be recorded at each observation.

5.2.2. The animals in the preliminary study (Section 4.1.) will be observed for a minimum of five days. The animals in the limit test (Section 4.2.) or the main study (Section 4.3.) will be observed for at least 14 days after dosing or, until all signs of reversible toxicity subside if this is beyond 14 days.

5.3. Local dermal irritation

Local dermal reactions at the treatment site will be assessed daily using the following numerical scoring system:

Erythema and eschar formation:

No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Oedema formation:

No oedema	0
Slight oedema	1
Well-defined oedema (area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure)	4

A note will be made of any lesion not covered by the above scoring system.

5.4. Bodyweight

The bodyweight of each rat in the limit test (Section 4.2.) or the main study (Section 4.3.) will be recorded on Days 1 (prior to dosing), 8 and 15 or at death. Where possible, group mean bodyweight data will be calculated.

6. Post-mortem examination

6.1. Surviving animals in the main study (Section 4.3.) or animals in the limit test (Section 4.2.) will be killed on Day 15 (unless delayed toxicity is observed and extension of the study is considered necessary to achieve the study objective).

6.2. All animals that die or are killed will be subjected to a macroscopic post-mortem examination which will consist of examination of the treated skin site and underlying tissues and opening the thoracic and abdominal cavities. The cranial cavity will also be examined if observations indicate neurotoxic activity. The macroscopic appearance or abnormal organs, when present, will be recorded.

6.3. Abnormal organs/tissues of animals surviving more than 24 hours may be preserved in suitably fixative pending any future requirement for microscopic pathological examination.

7. Statistical analysis

The LD_{50} value, and where possible, the value for males and females separately, will be calculated from the observed mortality data, using established procedures.

References:

Finney, D.J., (1971) Probit Analysis, 3rd edn., Cambridge University Press;

Weil, C.S. (1952) Biometrics, 8, 249;

Litchfield, J.T., and Wilcoxon, F., (1949) J. Pharmac. Exp. Ther., 96, 99.

8. Reports

Unless otherwise requested, six bound copies of the report will be issued. The report will include the following:

- A description of the test substance;
- The species/strain of the test animal;
- Full details of the experimental procedure used;

- A summary of observations on limit test or main study including tabulation of signs of reaction to treatment by sex and dose level and time of deaths;

- Bodyweight data;

- The calculated LD_{50} value, with associated 95 per cent confidence limits for all animals and, where possible, for males and females separately;

- Dose mortality curve(s), where appropriate;

- Autopsy findings.

9. Good laboratory practice

9.1 Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

United States Environmental Protection Agency, (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register, 17 August 1989.

Organisation for Economic Co-Operation and Development ISBN 92-64-12367-9, Paris 1982.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

9.2. Amendments to protocol

The Study Director will normally seek the approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3. Quality Assurance Department review

The Quality Assurance Department will:

(a) Conduct inspections of various phases of the study itself and/or of repetitive procedures relevant to the study design, and

(b) Audit the final report.

9.4. Maintenance of records

Following despatch of the final report, all raw data and other documents generated by HRC during the course of the study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

PROTOCOL

SKIN IRRITATION TO THE RABBIT

1. Introduction

Study objective:	Assessme . of skin irritation.
Protocol:	In compliance with: EEC methods for determination of toxicity, Directive 84/449/EEC (OJ No. L251, 19.9.84), Part B, Method B4 Acute toxicity (skin irritation).
Test species:	Rabbits of the New Zealand White strain.
Justification for selection of species:	Experience with this species and compliance with test guideline.
Route of administration:	Topical application.
Justification for route of administration:	Possible route of human exposure.
Duration of study:	Four days, with optional extension to assess reversibility/irreversibility of effects.

2. Test substance

2.1. The pH of the test substance may be measured. If the test substance is a powder or semi-solid it will be dissolved or suspended in distilled water and the pH measured. If the substance has a pH $\leq$ or $\geq$ 11.5 or has been shown to be highly toxic by the dermal route this study may not be performed.

2.2. If the test substance is a liquid, 0.5 ml will be applied to each treatment site.

2.3. If the test substance is a powder, solid or semi-solid, 0.5 g will be slightly moistened with water and applied to each treatment site.

2.4. The test substance will be stored at room temperature in non-continuous artificial light unless otherwise requested by the Sponsor on the Test Substance Data Sheet.

2.5. The absorption of the test substance will not be determined in this study.

2.6. Data concerning the stability, homogeneity and purity of the test substance is the responsibility of the Sponsor.

3. Animal management

3.1. A total of three healthy adult rabbits of the New Zealand White strain will be obtained from a commercial breeder and suitability acclimatized to the experimental environment.

3.2. The rabbits will be housed individually in metal cages with perforated floors.

3.3. Animals will have free access to tap water and SDS Stanrab (P) Rabbit diet or suitable alternative.

3.4. The batch of diet used for the study will not be analysed for chemical and microbiological contaminants.

3.5. Results of routine physical and chemical examination of drinking water at source (Grafham Final Water - Huntingdon North supply zone) as conducted usually weekly by the supplier, Anglian Water Services Ltd., are made available to Huntingdon Research Centre as quarterly summaries. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.6. Animal room temperature and relative humidity will be recorded daily. Thermostatic controls will be set to achieve a temperature of 20 ± 3° C and a relative humidity of 30-70 per cent RH.

3.7. Natural lighting will be supplemented with 12 hours of artificial light (0700-1900 hours) in each 24-hour period.

3.8. Each animal will be identified by a numbered aluminium tag placed through the edge of one ear. The identification number will be unique within the HRC Industrial Toxicology Department throughout the experimental period.

3.9. Each cage will be identified by a coloured label. Each label will display the study schedule number, animal number and the initials of the Study Director and Home Office licensee.

4. Treatment procedure

4.1. Preparation of animals

An area of skin approximately 10 cm x 10 cm on the dorso-lumbar region of each rabbit will be clipped free of hair.

4.2. Administration of test substance

The test substance will be introduced under a 2.5 cm x 2.5 cm gauze patch. The patch will be applied to one intact skin site on each rabbit. The trunk of the rabbit will then be bound with an elastic adhesive dressing, to maintain the test substance in intimate contact

with the skin for a period of four hours. The animals will not be restrained. At the end of the exposure period the dressing will be removed and the skin washed with water or suitable solvent to remove residual test substance.

5. Observations

5.1. Local dermal irritation

5.1.1. The animals will be observed 30 to 60 minutes and approximately 24, 48 and 72 hours following removal of the dressings. This observation period may be extended. This will not normally exceed 14 days after application.

5.1.2. The following numerical scoring system will be used:

Erythema and eschar formation:

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Oedema formation:

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

5.2. Clinical signs

Any observed signs of general toxicity and local effects other than those indicated above will be recorded.

6. Reports

Unless otherwise requested, six bound copies of the report will be issued. The report will include the following:

- A description of the test substance;
- The species/strain of test animal;
- Full details of the experimental procedures used;
- Tabulated numerical scores for each skin site at each observation;

- Narrative description of the nature and extent of irritation observed;

- Description of any toxic effects observed other than those represented by the numerical scoring system.

7. Good laboratory practice

7.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

United States Environmental Protection Agency, (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register, 17 August 1989.

Organisation for Economic Co-operation and Development ISBN 92-64-12367-9, Paris, 1982.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security, 1986, and subsequent revision, Department of Health, 1989.

7.2. Amendments to protocol

The Study Director will normally seek the approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

7.3. Quality Assurance Department review

The Quality Assurance Department will:

(a) Conduct inspections of various phases of the study itself and/or of certain repetitive procedures relevant to the study design, and

(b) Audit the final report.

7.4. Maintenance of records

Following despatch of the final report, all raw data and other documents generated by HRC during the course of this study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

PROTOCOL

EYE IRRITATION TO THE RABBIT

1. Introduction

Study objective:	Assessment of eye irritation
Protocol:	In compliance with: EEC Methods for determination of toxicity Directive 84/449/EEC (OJ No. L251, 19.9.84), Part B, Method B5 Acute toxicity (eye irritation)
Test species:	Rabbits of the New Zealand White strain.
Justification for selection of species:	Experience with this species and compliance with test guideline.
Route of administration:	Ocular instillation.
Justification for route of administration:	Possible route of human exposure.
Duration of study:	If injury persists, a maximum of 21 days after instillation.

2. Test substance

2.1. The pH of the test substance may be measured. If the test substance is a powder or semi-solid it will be dissolved or suspended in distilled water and the pH measured. If the substance has a pH $\leq$ 2 or $\geq$ 11.5 or has been shown to be a severe skin irritant, this study may not be performed.

2.2. If the test substance is a liquid, 0.1 ml will be placed undiluted into the lower everted lid of one eye.

2.3. If the test substance is powder, that weight (not to exceed 100 mg) which when gently compacted occupies a volume of 0.1 ml will be instilled into the lower everted lid of one eye. The weight will be recorded.

2.4. If the test substance is a solid or granular product, it will be ground to a fine dust and then treated as in 2.3. above.

2.5. If the test substance is in an aerosol container, it will be examined by spraying a single burst of one second duration at a distance of 10 cm directed at the open eye. The container will be weighed before and after use.

2.6. If the test substance is in a pump spray, the liquid will be expelled and 0.1 ml administered as for liquids.

2.7. The test substance will be stored at room temperature in non-continuous artificial light unless otherwise requested by the Sponsor on the Test Substance Data Sheet.

2.8. The absorption of the test substance will not be determined in this study.

2.9. Data concerning the stability, homogeneity and purity of the test substance is the responsibility of the Sponsor.

3. Animal Management

3.1. A total of three (nine if the effects of remedial irrigation are to be assessed) healthy adult rabbits of the New Zealand White strain will be obtained from a commercial breeder and suitable acclimatized to the experimental environment.

3.2. The rabbits will be housed individually in metal cages with perforated floors.

3.3. Animals will have free access to tap water and SDS Stanrab (P) Rabbit diet or suitable alternative.

3.4. The batch of diet used for the study will not be analysed for chemical and microbiological contaminants.

3.5. Results of routine physical and chemical examination of drinking water at source (Grafham Final Water - Huntingdon North supply zone) as conducted usually weekly by the supplier, Anglian Water Services Ltd., are made available to Huntingdon Research Centre as quarterly summaries. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.6. Animal room temperature and relative humidity will be recorded daily. Thermostatic controls will be set to achieve a temperature of 20 $\pm$ 3°C and a relative humidity of 30 - 70 per cent RH.

3.7. Natural lighting will be supplemented with 12 hours of artificial light (0700-1900 hours) in each 24-hour period.

3.8. Each animal will be identified by a numbered aluminium tag placed through the edge of one ear. The identification number will be unique within the HRC Industrial Toxicology Department throughout the experimental period.

3.9. Each cage will be identified by a coloured label. Each label will display the study schedule number, animal number and initial of the Study Director and Home Office licensee.

4. Treatment Procedure

4.1. Preparation of animals

Both eyes of all animals will be examined prior to instillation of the test substance to ensure there is no pre-existing corneal damage, iridial or conjuctival inflammation.

4.2. Administration of test substance

4.2.1. Initially one animal will be treated. If the ocular reactions are considered to be too severe by the Study Director the remaining two animals will not be treated.

4.2.2. The test substance will be administered using the method appropriate to its physical form (Section 2.). The lids will then be held together for one second before releasing. The second eye will remain untreated and serve as a control.

4.2.3. To investigate the effects of remedial irrigation, if considered necessary or requested by the Sponsor, the treated eye of each of three additional animals will be washed with water approximately 4 seconds after instillation and the treated eye of a further three animals approximately 30 seconds after instillation. Each eye will be washed for five minutes.

5. Observations

5.1. Local ocular irritation

5.1.1. The eyes of each animal will be examined and the grade of ocular reaction recorded, at 1 hour and at 1, 2 and 3 days after instillation. If considered necessary, additional observations will be made at 4, 7, 14 and 21 days to determine whether the reactions are of a transient or persistent nature.

5.1.2. For examination subsequent to Day 1 the eyes may be washed with sodium chloride injection B.P., 0.9 per cent w/v or distilled water. Any or all eyes may be further examined subsequent to Day 1 by application of fluorescein stain. One drop of flourescein sodium B.P., 2 per cent w/v is dropped directly onto the cornea, and excess reagent removed by flushing with sodium chloride injection B.P., 0.9 per cent w/v or distilled water.

5.1.3. The following numerical scoring system will be used:

Cornea

Opacity: degree of density (area most dense taken for reading)

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of the iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Nacreous area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4
Iris	
Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2
Conjuctivae	
Redness (refers to palpebral and bulbar conjunctivae, cornea and iris)	
Blood vessels normal	
Some blood vessels definitely hyperaemic (injected)	1
Diffuse, crimson colour, individual vessels not easily discernible	2
Diffuse beefy red	3
Chemosis (lids and/or nictitating membrane)	
No swelling	0
Any swelling above normal (includes nictitating membranes)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids more than half closed	4

5.2. Clinical signs

Any observed signs of general toxicity, and local effects other than those indicated above will be recorded.

6. Reports

Unless otherwise requested, six bound copies of the report will be issued. The report will include the following:

- A description of the test substance, including pH where applicable;
- The species/strain of test animal;
- Full details of the experimental procedures used;
- Tabulated numerical scores for each animal at each observation;
- Narrative description of the nature and extent of irritation observed;
- Description of any toxic effects observed, other than those represented by the numerical scoring system.

7. Good Laboratory Practice

7.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

United States Environmental Protection Agency, (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Organisation for Economic Co-operation and Development. ISBN 2-64-12367-9, Paris 1982.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

7.2. Amendments to protocol

The Study Director will normally seek the approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

7.3. Quality Assurance Department review

The Quality Assurance Department will:

(a) Conduct inspections of various phases of the study itself and/or of certain repetitive procedures relevant to the study design, and

(b) Audit the final report.

7.4. Maintenance of records

Following despatch of the final report, all raw data and other documents generated by HCR during the course of this study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

PROTOCOL

SKIN SENSITIZATION IN THE GUINEA-PIG

1. Introduction

Study objective:	Assessment of potential to produce delayed contact hypersensitivity.
Protocol:	In compliance with: EEC Methods for determination of toxicity Directive 84/449/EEC (OJ No. L251, 19.9.84), Part B, Method B6 Acute toxicity (skin sensitization).
Test species:	Albino guinea-pigs of the Dunkin/Hartley strain.
Justification for selection of species:	Experience with this species and compliance with test guideline.
Route of administration:	Intradermal injection and topical application.
Justification for route of administration:	Methodology requirement.
Duration of study:	Five to six weeks.

2. Test substance

2.1. The test substance, when formulated, will be prepared in a suitable vehicle at the required concentrations (see preliminary study section 4.1. below). Suitable vehicles for use would be distilled water, physiological saline, liquid paraffin B.P., acetone and Alembicol D (a medium chain triglyceride obtained from coconut oil).

2.2. The test substance, when formulated, will be prepared on the day of dosing.

2.3. The test substance will be stored at room temperature in non-continuous artificial light unless otherwise requested by the Sponsor on the Test Substance Data Sheet.

2.4. The absorption of the test substance will not be determined in this study.

2.5. Data concerning the stability, homogeneity, and purity of the test substance is the responsibility of the Sponsor.

2.6. Unless the Sponsor indicates that the test substance is physically or chemically unstable in the proposed vehicles, assays of dose formulations will not be undertaken at HRC.

3. Animal management

3.1. Thirty healthy female nulliparous and non-pregnant albino guinea-pigs, of the Dunkin/Hartley strain will be obtained from a commercial breeder. The guinea-pigs will weigh approximately 350 g on arrival and be six to seven weeks old. All guinea-pigs will be suitably acclimatized to the experimental environment.

3.2. The bodyweight of each guinea-pig will be recorded at the start and completion of the study.

3.3. The animals will be allocated to groups, without conscious bias, as follows:

Group	Number of guinea-pigs
Control	10
Test	20

3.4. The guinea-pigs will be housed in groups of 5 to 10 in suspended metal cages with wire mesh floors.

3.5. Animals will have free access to tap water and a vitamin C-enriched guinea-pig Diet F.D.1. or suitable alternative and hay once weekly.

3.6. The batch of diet used for the study will not be analysed for chemical or microbiological contaminants.

3.7. Results of routine physical and chemical examination of drinking water at source (Grafham Final Water - Huntingdon North supply zone) as conducted usually weekly by the supplier, Anglian Water Services Ltd., are made available to Huntingdon Research Centre as quarterly summaries. The analytical data will be lodged in the Huntingdon Research Centre Archives.

3.8. Animal room temperature and relative humidity will be recorded daily. Thermostatic controls will be set to achieve a temperature of 22 ± 3° C and a relative humidity of 30 - 70 per cent RH.

3.9. Natural lighting will be supplemented with 12 hours of artificial light (0700-1900 hours) in each 24-hour period.

3.10. Each animal will be individually identified by ear tattoo or tag.

3.11. Each cage will be identified by a coloured label. Each label will display the study schedule number, animal numbers and the initials of the Study Director and Home Office licensee.

4. Treatment procedure

4.1. Preliminary irritation study

4.1.1. The objective of these investigations is to identify irritant test substance concentrations, when practicable, for the induction phase of the main study. In addition, suitable non-irritant concentrations of the test substance, by the topical route of administration, will be identified for the challenge application.

Details of the preliminary work will be reported.

Animals for these investigations will be additional to those described in section 3. above.

The procedure employed for these investigations will be as follows:

4.1.2. Intradermal injections

Intradermal injections (0.1 ml/site) will be made into the clipped flank of two guinea-pigs, using a range of concentrations of the test substance in a suitable vehicle. The resulting dermal reactions will be assessed approximately 24 hours later and where necessary 72 hours later.

The following parameters will be recorded:

Diameter (D) in mm, Erythema (E) and Oedema (O) using the following numerical scoring system.

Erythema and eschar formation:

No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Oedema formation:

No oedema	0
Sight oedema	1
Well-defined oedema (edges of area well-defined by definite raising	2
Moderate oedema (raised approximately 1 millimeter)	3
Severe oedema (raised more than 1 millimeter and extending beyond the area of exposure)	4

4.1.3. Topical application

Patches of Whatman No. 3 paper (2 cm x 2 cm) will be saturated with a range of concentrations of the test substance in a suitable vehicle and applied to the clipped and shaved flanks of each of four guinea-pigs. The patches will be covered by a strip of "Blenderm"* and firmly secured by "Elastoplast"* wound round the trunk and covered with "Sleek"* impervious plastic adhesive tape. The dressings will be removed after an exposure period of approximately 24 hours and the reaction sites will be assessed for erythema and oedema on a numerical basis according to the scale described in section 4.1.2. Further examination of the sites will be carried out approximately 24 and 48 hours after removal of the dressings.

4.2. Selection of concentrations of test substance for main study

4.2.1. Selection of the concentration of test substance will be based on the following criteria:

4.2.2. Induction injection - concentration that will produce slight irritation when injected intradermally but will not adversely affect the animals.

4.2.3. Topical induction - concentration that will produce some irritation but not adversely affect the animals.

4.2.4. Topical challenge - the maximum non-irritant concentration and a dilution of this.

4.2.5. Selection of these concentrations may however depend on a number of other factors and exact criteria do not always apply. These factors include (i) slope of dose response curve and (ii) experience with similar test substances.

4.2.6. Prior to dose administration the Study Director will authorize the choice of dose levels in the raw data.

4.3. Main study

4.3.1. Induction

Intradermal injections

An area of dorsal skin from the scapular region (approximately 4 x 6 cm) will be clipped free of hair. Three pairs of intradermal injections (0.1 ml/site) will be made just within the boundaries of a 2 x 4 cm area in the clipped region as follows:

(1) Freund's complete adjuvant 50:50 with water for irrigation.

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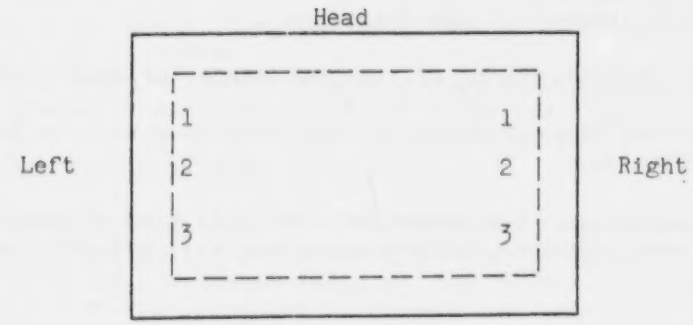
* or suitable alternatives.

(2) The test substance at the concentration selected in the preliminary study in a suitable vehicle.

(3) The test substance at the concentration used in (2), emulsified in 50:50 mixture of Freund's complete adjuvant and the vehicle used in (2).

The position of the intradermal injections in the scapular region is shown below:



_____ clipped area

_ _ _ _ _ area in which injections will be made

Topical applications

One week after the injections the same scapular area will be clipped and shaved free of hair. A 2 cm x 4 cm patch of Whatman No. 3 paper will be saturated with the test substance at the selected concentration in a suitable vehicle and placed over the injection sites of the test animals. The patch will be covered by a length of Blenderm"* and firmly secured by "Elastoplast"* wound round the torso of the animal and secured with "Sleek"* impervious plastic adhesive tape. The dressings will be left in place for approximately 48 hours.

In the absence of skin irritation during the topical application in the preliminary studies, the site of the topical induction application will be pre-treated, by gently rubbing with 0.2 ml/site 10 per cent w/w sodium lauryl sulphate in petrolatum, 24 hours before treatment with the test substance. No bandages will be applied.

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* or suitable alternative.

During the induction period, the guinea-pigs of the control group will receive similar intradermal injections and topical applications with the exception that the test substance will be omitted.

4.3.2. Challenge

The test and control guinea-pigs will be challenged two weeks after the topical induction application.

Hair will be removed by clipping and shaving from an area on the left flank of each guinea-pig. A 2 cm x 2 cm patch of Whatman No. 3 paper will be saturated with a suitable concentration of the test substance and applied to an anterior site on the flank. A further concentration of the test substance will be applied in a similar manner to a posterior site. The patches will be covered using the dressing described in section 4.1.3. above. The dressings will be removed after approximately 24 hours. The sites will be assessed for erythema and oedema approximately 24, 48 and 72 hours after removal of the dressings using the arbitrary scale described in 4.1.2. Where necessary the sites may be rinsed with water or a suitable solvent to remove residual test substance.

4.3.3. Re-challenge

A second challenge may be carried out approximately one week after the first challenge. The method will be similar to that described for the first challenge with the exception that the right flanks of all the guinea-pigs will be used.

4.3.4. Interpretation

Dermal reactions in the test animals elicited by the challenge application(s) will be compared with the findings simultaneously obtained in the control animals.

A test animal will be considered to show positive evidence of delayed contact hypersensitivity if the observed dermal reaction at challenge is definitely more marked and/or persistent than the maximum reaction seen in animals of the control group.

If the dermal reaction seen in a test animal at challenge is slightly more marked and/or persistent than (but not clearly distinguishable from) the maximum reaction seen in control animals, the result for that test animal will be classified as inconclusive.

A test animal will be considered to show no evidence of delayed contact hypersensitivity if the dermal reaction resulting from the challenge application(s) is the same as, or less marked and/or persistent than the maximum reaction seen in animals of the control group.

4.3.5. Positive control

The sensitivity of the guinea-pig strain used is checked periodically at HRC with formalin, a known sensitizer.

5. Reports

Unless otherwise requested, six bound copies of the report will be issued. The report will include the following:

- A description of the test substance;
- The species/strain of test animal;
- Full details of the experimental procedures used;
- The numerical scores following the challenge application;
- Assessment of the result for each animal;
- Individual bodyweights at both the start and completion of the study;
- Summary of recent positive control data.

6. Good laboratory practice

6.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

United States Environmental Protection Agency (TSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Organisation for Economic Co-operation and Development. ISBN 92-64-12367-9, Paris 1982.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Good Laboratory Practice, the United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

6.2. Amendments to protocol

The Study Director will normally seek the approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal

welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

6.3. Quality Assurance Department Review

The Quality Assurance Department will:

(a) Conduct inspections of various phases of the study itself and/or of certain repetitive procedures relevant to the study design, and

(b) Audit the final report.

6.4. Maintenance of records

Following dispatch of the final report, all raw data and other documents generated by HRC during the course of this study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd., Archive, Huntingdon, United Kingdom.

ANNEX II

PROTOCOL

ACUTE TOXICITY TO RAINBOW TROUT

1.	Introduction	
1.1.	Study objective	To determine the acute toxicity (LC_{50}) of the test substance when dispersed in water.
1.2.	Test species	Rainbow trout (Oncorhynchus mykiss)
1.3.	Purpose	In order to aid in the assessment of the toxic effect of the test substance in the aquatic environment, this study has been prepared following the recommendations of the OECD Guideline for Testing of Chemicals No. 203, "Fish, Acute Toxicity Test". The study follows guidelines laid down in EEC Directive 67/548/EEC Annex V Part C.1. "Acute Toxicity for Fish" as published in EEC Directive 84/449/EEC.
1.4.	Selection of species	The rainbow trout, Oncorhynchus mykiss, has been selected following recommendations in OECD Guideline No. 203 and EEC Directive 84/449/EEC.
1.5.	Route of administration	The dispersal of the test substance in the surrounding medium is considered to represent the most probable route of exposure in the environment.
1.6.	Duration of study	96 hours.
1.7.	Dates of study	Actual dates to be advised by Protocol Amendment.
2.	Test substance	
2.1.	Preparation	Once at the start of the study (daily/continuously in the case of semi-static/continuous flow tests) the test substance will be dispersed in water with the aid of auxiliary solvents/surfactants where necessary (at a maximum of 100 µl/l final volume). Details to be advised by Protocol Amendment.

2.2.	Storage	The test substance will be stored at room temperature under normal laboratory lighting unless otherwise requested by the Sponsor.
2.3.	Absorption	Absorption is via the gills and other membranes exposed to the surrounding water. Specific determination of absorption will not be made in this study.
2.4.	Stability	Data concerning the stability, homogeneity and analytical purity of the test substance, as provided, is the responsibility of the Sponsor.
3.	Test organisms	
3.1.	Species	Rainbow trout (Oncorhynchus mykiss)
3.2.	Source	Westacre Trout Farm, Norfolk, United Kimgdom
3.3.	Number	Approximately 100.
3.4.	Size	4 - 6 cm.
3.5.	Age	$\leq$ 1 year.
3.6.	Sex	Not relevant to this study.
4.	Acclimatization	
4.1.	Duration	At least 12 days. At least seven days in water of the quality to be used in the test.
4.2.	Vessels	Glass or fibreglass tanks providing at least 1 litre of tank volume per fish.
4.3.	Medium	Laboratory tap water, dechlorinated by the addition of sodium thiosulphate, or reconstituted water as appropriate. When "single-pass" systems are not possible, the water will be recirculated via activated carbon/gravel-bed purification filters.
4.4.	Photoperiod	16 h light: 8 h dark.
4.5.	Temperature	13 - 17 °C.
4.6.	Aeration	To give $\geq$ 80 per cent air saturation value.
4.7.	Feeding	Commercial trout pellets to repletion at least five times per week. Feeding is discontinued 24 h prior to testing.

4.8.	Mortality	Following a 48 h settling-in period, mortalities are recorded and the following criteria applied: (i) › 10% - rejection of entire batch (ii) 5-10% in 7 days - acclimatization continued for seven additional days (iii) ‹ 5% in 7 days - acceptance of batch.
5.	Exposure	
5.1.	Duration	96 hours.
5.2.	Vessels	(i) Glass aquaria containing 20-40 l test medium for static and semi-static tests (depending on "5.3 Loading"). (ii) Glass aquaria containing 20 l test medium for continuous flow tests.
5.3.	Loading	Not greater than 1 g fish per litre for static and semi-static tests. This loading may be exceeded in continuous flow tests.
5.4.	Animals per test concentration	10 fish.
5.5.	Test concentrations	5, logarithmically spaced by a factor not exceeding 1.8, plus 1 control. The actual test concentrations are assigned according to preliminary range-finding tests, using two to five fish at each concentration in a series spanning several orders of magnitude. Details to be advised by Protocol Amendment.
5.6.	Negative control	Water with no test substance included. Where auxiliary solvents/surfactants are employed, the control fish will also be exposed to a concentration equal to that employed in the highest concentration of the test series.
5.7.	Positive control	Not included in this study.

5.8.	Verification of test concentrations	Details to be advised by Protocol Amendment.
5.9.	Frequency of exposure	Continuous.
5.10.	Renewal of test media (as specified by Protocol Amendment)	Static test: Test media are prepared at the start of the test and are not renewed. Semi-static test: Test media are renewed daily. Continuous flow test: Test media are renewed continuously at a rate of 70-140 ml/minute.
5.11.	Diluent (as specified by Protocol Amendment)	(a) Laboratory tap water dechlorinated by the addition of sodium thiosulphate. Hardness approximately 350 mg/l (as $CaCO_3$), pH 6-8.5. (b) Reconstituted water (see Notes 1 and 2 below).
5.12.	Photoperiod	16 h light : 8 h dark.
5.13.	Temperature	$\pm$ 1 °C within the range 13-17 °C.
5.14.	Aeration	Via narrow bore glass tubes positioned in each aquarium to give $\geq$ 70 per cent of the air saturation value. The diluent stock only is aerated in continuous flow tests.
5.15.	Feeding	None during test period.
5.16.	Identification	Fish are not individually identified.
5.17.	Randomization	Fish are selected at random from the total number available. No other method of randomization is used.

6. Observations

6.1. The fish are inspected at least after 3, 24, 48, 72 and 96 hours and at intermediate times when appropriate. Mortalities are recorded and any abnormalities in appearance or behaviour shown by surviving fish are summarized. Criteria of death are absence of respiratory movement and lack of response to external stimulation.

6.2. Measurements of pH, dissolved oxygen and temperature in each test vessel are recorded daily.

7. Results

7.1. The 3, 24, 48, 72, 98-hour LC_{50} values and associated 95 per cent confidence limits are calculated using established procedures suitable for the data e.g. Finney, D.J. (1971) Probit Analysis (3rd Edition) Cambridge University Press; Litchfield, J.T. and Wilcoxon, F. (1949) J. Pharmac. Exp. Ther. 96, 99; Thompson, W.R. and Weil, C.S. (1952) Biometric 8, 51-54.

8. Reports

8.1. An advance copy of the report will be issued for comment by the Sponsor prior to printing the final report. Unless otherwise requested, six bound copies of the report will be issued. The report will include the following information:

- A description of the test substance;
- The species of animal;
- Details of the experimental procedures followed;
- A summary of observations and environmental parameters;
- The maximum concentration causing no mortality after 96 hours;
- The minimum concentration causing 100 per cent mortality after 96 hours;
- The cumulative mortality at each concentration after 24, 48, 72 and 96 hours;
- The calculated LC_{50} values and 95 per cent confidence limits after 96 hours and, where possible, after 24, 48 and 72 hours;
- The concentration-mortality curve at 96 hours;
- The toxicity curve (log LC_{50} v log time) where appropriate;
- Any available information on the concentration of test substance in the test solutions.

9. Good laboratory practice

9.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 (FIFRA) and 792 (TSCA) Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9 Paris 1982.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

9.2. Amendments to protocol

The Study Director will normally seek approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3. Quality Assurance Unit review

The Quality Assurance Unit will:

(a) Conduct inspections of various phases of the study itself and/or of repetitive procedures relevant to the study design, and

(b) Audit the final report.

9.4. Maintenance of records

Following despatch of the final report, all raw data and other documents generated by HRC during the course of the study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

10. Notes

(1) Reconstituted Water (EEC/OECD)

All chemicals should be of analytical grade. The water is purified by reverse osmosis to produce a water of conductivity less than 5 $uScm^{-1}$.

Stocks solutions

(i)	$CaCl_2.2H_2O$	117.6 g/l
(ii)	$MgSO_4.7H_2O$	49.3 g/l
(iii)	$NaHCO_3$	25.9 g/l
(iv)	KCl	2.3 g/l

Mix 2.5 ml of solutions (i) - (iv) and make up to 1 litre with water. Adjust pH to 7.9 $\pm$ 0.3 with NaOH or HCl as necessary. The water should be aerated for at least 12 hours prior to use.

(2) Reconstituted Water (UK Control of Pesticide Regulations 1986)

The dilution water is prepared by addition of the stock solutions specified below to reverse osmosis purified water.

(a)	$CaCl_2.6H_2O$	320	g
	NaCl	29	g
	$NaNO_3$	9	g
	+ water to 1 litre		
(b)	$MgSO_4.7H_2O$	151	g
	Na_2SO_4	79	g
	+ water to 1 litre		
(c)	$NaHCO_3$	27.5	g
	+ water to 1 litre		

To each 100 litres of water are added 10 ml of solutions (a) and (b) and 100 ml of solution (c).

PROTOCOL

ACUTE TOXICITY TO DAPHNIA

1.	Introduction	
1.1.	Study objective	To determine the acute toxicity (EC_{50}) of the test substance when dispersed in water.
1.2.	Test species	Daphnia magna (Straus)
1.3.	Purpose	In order to aid in the assessment of the toxic effect of the test substance in the aquatic environment, this study has been prepared following the recommendations of the OECD Guideline for Testing of Chemicals No. 202, "Daphnia sp., 14 days Reproduction Test (including an Acute Immobilization Test)", Phase I. The study follows guidelines laid down in EEC Directive 67/548/EEC, Annex V, Part C.2, "Acute Toxicity to Daphnia" as published in EEC Directive 84/449/EEC.
1.4.	Selection of species	Daphnia magna has been selected following recommendations in OECD Guideline No. 202 and EEC Directive 84/449/EEC.
1.5.	Route of administration	The dispersal of the test substance in the surrounding medium is considered to represent the most probable route of exposure in the environment.
1.6.	Duration of study	48 hours.
1.7.	Dates of study	Actual dates to be advised by Protocol Amendment.
2.	Test substance	
2.1.	Preparation	The test substance is dissolved/dispersed in the test water once at the start of the study. If auxiliary solvents or surfactants are used to aid in the dispersion, they will be used at a maximum concentration of 100 µl/l (final volume). Details to be advised by Protocol Amendment.
2.2.	Storage	The test substance will be stored at room temperature under normal laboratory lighting unless otherwise requested by the Sponsor.

2.3.	Absorption	Absorption is via the membranes exposed to the surrounding water. Specific determination of absorption will not be made in this study.
2.4.	Stability	Data concerning the stability, homogeneity and analytical purity of the test substance, as provided, is the responsibility of the Sponsor.
3.	Test organisms	
3.1.	Species	Daphnia magna (Straus)
3.2.	Source	Derived from a laboratory strain supplied by the Institute National de Recherche Chimique Appliquée (I.R.CH.A.).
3.3.	Number	Approximately 400.
3.4.	Size	Not applicable to this study.
3.5.	Age	First instar.
3.6.	Sex	Females, produced by parthenogenesis.
4.	Acclimatization/culture	
4.1.	Duration	A parthenogenetically reproducing population is maintained in the laboratory.
4.2.	Vessels	2 to 3-litre glass or polypropylene beakers stocked at 50-100 daphnids per vessel initially.
4.3.	Medium	Laboratory tap water, dechlorinated by the addition of sodium thiosulphate and "aged" by recirculation through a gravel filter-bed.
4.4.	Photoperiod	16 h light: 8 h dark.
4.5.	Temperature	20 $\pm$ 2° C.
4.6.	Aeration	None.
4.7.	Feeding	0.2 ml Liquifry No. 2 fry food (the Liquifry Co. Ltd., Dorking) per vessel per working day plus a suspension of a cultured Scenedesmus and Selenastrum sp. when available.

4.8.	Collection of first instars	Brood females are removed to a separate culture vessel the day before the test. The following day the first instars produced overnight are used for testing.
5.	Exposure	
5.1.	Duration	48 hours.
5.2.	Vessels	250-300 ml glass beakers containing 200 ml of test solution. Covered with aluminium foil to reduce evaporation.
5.3.	Loading	10 first instars per vessel. Volatile chemicals will be tested in completely filled and stoppered vessels with a loading of no more than 20 daphnids per litre.
5.4.	Animals per test concentration	20 instars.
5.5.	Test concentrations	9, logarithmically spaced by a factor not exceeding 1.8, plus 1 control, each in duplicate. The actual test concentrations are assigned according to preliminary range-finding tests, and will be given in the Study Details sheet.
5.6.	Negative control	Water with no test substance included. Where auxiliary solvents/surfactants are used, the control daphnids will also be exposed to a concentration equal to that employed in the highest concentration of the test series.
5.7.	Positive control	Not included in this study.
5.8.	Verification of test concentrations	Details to be advised by Protocol Amendment.
5.9.	Frequency of exposure	Continuous.
5.10.	Renewal of test media	None.
5.11.	Diluent	Dechlorinated and aged laboratory tap water. Hardness approximately 300 mg/l pH 6.5-8.0
5.12.	Photoperiod	16 h light: 8 h dark (subdued light).
5.13.	Temperature	20 $\pm$ 1° C.

5.14.	Aeration	None. The oxygen concentration should remain $\geq$ 70 per cent ASV at the end of the test.
5.15.	Feeding	None during test period.
5.16.	Identification	Animals are not individually identified.
5.17.	Randomization	Animals are selected at random from the total number collected. No other method of randomization is used.

6. Observations

6.1. The number of Daphnia which are unable to swim for approximately 15 seconds after gentle agitation of each vessel is recorded at 24 hours and 48 hours. Temperature is recorded daily and pH and dissolved oxygen measurements are made for each vessel at the start and at the end of the study.

7. Results

7.1. The 24-hour and 48-hour EC_{50} (immobilization) values and associated 95 per cent confidence limits are calculated using established procedures suitable for the data, e.g. Finney, D.J. (1971), Probit Analysis (3rd Edition), Cambridge University Press; Litchfield, J.T. and Wilcoxon, F. (1949), J. Pharmac. Exp. Ther. 96, 99; Thompson, W.R. and Weil, C.S. (1952), Biometrics 8, 51-54.

8. Reports

8.1. An advance copy of the report will be issued for comment by the Sponsor prior to printing the final report. Unless otherwise requested, six bound copies of the report will be issued. The report will include the following information:

- A description of the test substance;
- Details of the experimental procedures used;
- A summary of observations and environmental parameters;
- The maximum concentration causing no immobilization after 48 hours;
- The minimum concentration causing 100 per cent immobilization after 48 hours;
- The cumulative immobilization at each concentration after 24 and 48 hours;
- The calculated EC_{50} values and 95 per cent confidence limits at 24 and 48 hours;

- The concentration-immobilization curve at 24 and 48 hours;

- Any available information on the concentration of test substance in the test solutions.

9. Good laboratory practice

9.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 (FIFRA) and 792 (TSCA) Federal Register, 29 November 1983 and subsequent amendment Federal Register, 17 August 1989.

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris, 1982.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

9.2. Amendments to protocol

The Study Director will normally seek approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3. Quality Assurance Unit review

The Quality Assurance Unit will:

(a) Conduct inspections of various phases of the study itself and/or of repetitive procedures relevant to the study design, and

(b) Audit the final report.

9.4. Maintenance of records

Following dispatch of the final report, all raw data and other documents generated by HRC during the course of the study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

PROTOCOL

ACTIVATED SLUDGE, RESPIRATION INHIBITION TEST

1.	Introduction	
1.1.	Study objective:	To assess the effect of a test substance on the respiratory rate of micro-organisms.
1.2.	Test species:	A mixed population of activated sludge micro-organisms.
1.3.	Purpose:	In order to aid in the assessment of the toxic effect of the test substance in the aquatic environment, this study has been prepared following the recommendations of the OECD Guideline for Testing of Chemicals No. 209, "Activated Sludge, Respiration Inhibition Test". The study follows the United Kingdom Health and Safety Commission "Approved Code of Practice for the Notification of New Substances Regulations 1982 Methods for the Determination of Ecotoxicity" EEC Directive 79/831 as published in the EEC Directive 67/548 Annex VIII Part C (87/302/EEC).
1.4.	Selection of species:	Activated sludge micro-organisms have been selected following recommendations in OECD Guideline No. 209 and EEC Directive 87/302.
1.5.	Route of administration:	The dispersal of the test substance in the surrounding medium is considered to represent the most probable route of exposure in the environment.
1.6.	Duration of study:	3-hours contact time.
1.7.	Dates of study:	Actual dates to be advised by Protocol Amendment.
2.	Test substance	
2.1.	Preparation:	Once at the start of the study, the test substance will be dispersed in water without the aid of auxiliary solvents/surfactants unless absolutely necessary (at a maximum of 100 ul/l final volume). (See Study Details Sheet.) Details to be advised by Protocol Amendment.

2.2.	Storage:	The test substance will be stored at room temperature under normal laboratory lighting unless otherwise requested by the Sponsor.
2.3.	Absorption:	Not applicable.
2.4.	Stability:	Data concerning the stability, homogeneity and analytical purity of the test substance as provided is the responsibility of the Sponsor.

3. Test organisms

3.1.	Species:	A mixed population of activated sludge micro-organisms.
3.2.	Source:	The aeration stage of the HRC sewage treatment plant treating predominantly domestic sewage.
3.3.	Preparation of inoculum:	The sample of activated sewage sludge is aerated continuously at 20 ± 2° C and fed 50 ml synthetic sewage per litre per day (max. four days) if not used on the day of collection. Before use:

(i) A small sample of known volume is dried and weighed in order to allow the preparation of a sludge with a suspended solids level of 4 g/l (± 10 per cent) or equivalent to 1.6 g/l in the final test culture. The sample may be diluted or filtered and resuspended (dechlorinated tap water) as appropriate.

(ii) The pH is buffered to pH 6.0-8.0 with sodium bicarbonate solution.

4. Exposure

4.1.	Contact time:	3 hours.
4.2.	Vessels:	500 ml glass conical flasks.
4.3.	Water:	Laboratory tap water dechlorinated by sodium thiosulphate (hardness approx. 300 mg/l as $CaCO_3$, pH 6-8.5).
4.4.	Aeration:	Compressed air via narrow bore glass tubes (approx. 0.5-1 litre/min.).

4.5.	Measuring apparatus:	280 ml darkened glass BOD bottle fitted with a dissolved oxygen probe (Yellow Springs Instruments Model No. 57).
4.6.	Synthetic sewage:	16 g peptone 11 g meat extract 3 g urea 0.7 g NaCl 0.4 g $CaCl_2.2H_2O$ 0.2 g $MgSO_4.7H_2O$ 2.8 g K_2HPO_4 - dissolved in 1 litre tap water.
4.7.	Test concentrations:	5, logarithmically spaced by a factor of typically 2 but not exceeding 3.2. The actual test series will be given in a Protocol Amendment following range-finding tests.
4.8.	Reference substance:	3,5-dichlorophenol at concentrations 3.2, 10 and 32 mg/l.
4.9.	Temperature:	20 ± 2° C.
4.10.	Photoperiod:	Normal laboratory lighting.
4.11.	Control:	Inoculated medium without the test substance.

5. Procedure

5.1. At the time "0" 16 ml of synthetic sewage is made up to 300 ml with water in a 1 litre beaker. 200 ml of microbial inoculum are added and the mixture aerated at approximately 0.5-1 litre per minute. Thereafter, at 15 minute intervals, the procedure is repeated with the appropriate amounts of test or reference substance solutions being added prior to making up to 300 ml and adding the inoculum.

The order will be Control 1; Reference 1, 2, 3; Test Substance 1, 2, 3, 4, 5; Control 2.

5.2. As each vessel reaches 30 minutes contact time, the contents are poured into the measuring vessel and the rate of respiration measured between approx. 6.5 mg O_2/l and 2.5 mg O_2/l or for a 10 minute period when the respiration rate is low. A clean measuring vessel is used for the Control group, the Reference group and the Test Substance group. Within each group the lowest test concentrations are measured first. The contents of the measurement vessel are returned to the contact vessel after measurement.

5.3. The procedure is repeated after a three-hour contact period.

6. Observations

6.1. Oxygen consumption for each culture after contact times of 30 minutes and 3 hours is measured and recorded by chart.

7. Results

7.1. The two respiration rates for each culture are calculated from the linear section of the recorder trace (approx. 6.5 mg O_2/l - 2.5 mg O_2/l).

7.2. The inhibitory effect for each concentration at each contact time is calculated from the equation

$$\% \text{ inhibition} = 1 - \frac{2Rs}{Rc_1 + Rc_2} \times 100$$

Where Rs = oxygen consumption rate for test and reference samples.

$Rc_1 + Rc_2$ = oxygen consumption rates for Controls 1 and 2.

7.3. Per cent inhibition is plotted against concentration on log-normal or log-probability paper and the EC_{50} value derived. Values will be rounded to the first significant figure.

7.4. The results are valid if:

(i) The two control respiration rates are within 15 per cent of each other.

(ii) The EC_{50} (3 h contact) for 3,5-dichlorophenol lies within the range 5-30 mg/l.

8. Reports

8.1. An advance copy of the report will be issued for comment by the Sponsor prior to printing the final report. Unless otherwise requested six bound copies of the report will be issued. The report will include the following information:

- A description of the test and reference substances;
- The test inoculum;
- The experimental procedure and conditions;
- The measured oxygen consumption rates for all vessels after 30-minute and 3-hour contact times;
- The calculated percentage inhibition values for each test and reference concentration at 30 minutes and 3 hours;

- The derived 30-minutes and 3-hour EC_{50} values for the test and reference substances.

9. Good laboratory practice

9.1. Regulations:

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 (FIFRA) and 792 (TSCA) Federal Register, 29 November 1983 and subsequent amendment Federal Register, 17 August 1989.

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

9.2. Amendments to protocol

The Study Director will normally seek approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3. Quality Assurance Unit review:

The Quality Assurance Unit will:

(a) Conduct inspections of various phases of the study itself and/or of repetitive procedures relevant to the study design, and

(b) Audit the final report.

9.4. Maintenance of records:

Following dispatch of the final report, all raw data and other documents generated by HRC during the course of the study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

PROTOCOL

RAPID DETERMINATION OF THE ALGISTATIC EFFECT OF CHEMICALS

1. Introduction

1.1. Study objective

To determine the concentration of the test substance which results in an increase of the algel cell density which is 50 per cent of that shown by the untreated culture (i.e., the EC_{50} value).

1.2. Test species

One of the following species (to be advised by Protocol Amendment):

(i) Chlorella vulgaris (Strain No. CCAP 211/11b);

(ii) Selenastrum capricornutum (Strain No. CCAP 278/4;

(iii) Scenedesmus subspicatus (Strain No. CCAP 276/20).

1.3. Purpose

In order to aid in the assessment of the toxic effect of the test substance in the aquatic environment, this study has been prepared following the recommendations of the OECD Guideline for Testing of Chemicals No. 201, "Algal Growth Inhibition Test".

The study follows the United Kingdom Health and Safety Commission "Approved Code of Practice for the Notification of New Substances Regulations 1982 Methods for the Determination of Ecotoxicity", EEC Directive 79/831, as published in the EEC Directive 67/548, Annex VIII, Part C (87/302/EEC).

1.4. Selection of species

The green algae, Chlorella vulgaris, Selenastrum capricornutum and Scenedesmus subspicatus have been selected following recommendations in OECD Guideline No. 201 and EEC Directive 87/302. The choice of test species will be made by the Study Director if no preference is indicated by the Sponsor.

1.5. Route of administration

The dispersal of the test substance in the surrounding medium is considered to represent the most probable route of exposure in the environment.

1.6.	Duration of study	72 hours.
1.7.	Dates of study	Actual dates to be advised by Protocol Amendment.
2.	Test substance	
2.1.	Preparation	Once at the start of the study, the test substance will be dispersed in the nutrient medium with the aid of auxiliary solvents/surfactants where necessary (at a maximum of 100 µl/l final volume). Details to be advised by Protocol Amendment.
2.2.	Storage	The test substance will be stored at room temperature under normal laboratory lighting unless otherwise requested by the Sponsor.
2.3.	Absorption	Absorption is via the total cell membrane exposed to the surrounding water. Specific determination of absorption will not be made in this study.
2.4.	Stability	Data concerning the stability, homogeneity and analytical purity of the test substance, as provided, is the responsibility of the Sponsor.
3.	Test organisms	
3.1.	Species	(i) Chlorella vulgaris Strain No. CCAP 211/11b; (ii) Selenastrum capricornutum Strain No. CCAP 278/4. (iii) Scenedesmus subspicatus Strain No. CCAP 276/20.
3.2.	Source	Culture Centre of Algae and Protozoa, c/o The Freshwater Biological Association, Cumbria, United Kingdom.
4.	Algal culture	
4.1.	Stock Culture	Samples of the master culture are used to inoculate 100 ml volumes of sterile nutrient media (see 10. Notes) contained in conical flasks. The flasks are capped and kept under constant illumination at 23 ± 2° C for approximately one week. If

		not used immediately the cultures can be kept for up to two months before their transfer to fresh nutrient is required.
4.2.	Pre-culture	Sufficient stock culture is added to 1 to 2 litres of sterile medium to give an initial cell density of approximately 10^4 cells/ml. Culture of this suspension continues for a further four to five days or until the algal cell density is approximately 2×10^4-10^5 cells/ml. Dilution may be required to obtain this density immediately prior to testing.

4.3. The culture should be maintained under sterile conditions and the flasks shaken at least once per day to prevent settlement (alternatively the cultures may be aerated or agitated by orbital shaker continuously).

5. Exposure

5.1.	Duration	72 hours. The study may be extended to 96 hours at the request of the Sponsor.
5.2.	Vessels	250 ml conical flasks (capped).
5.3.	Inoculum	50 ml algal pre-culture (approximately 2×10^4-10^5 cells/ml) to 50 ml nutrient medium containing the test substance. The resulting test culture should have an initial absorbance value of 0.02-0.05 at 665 nm.
5.4.	Test concentrations	5 logarithmically spaced concentrations plus control (each in triplicate) plus blank. The actual test concentrations are assigned according to preliminary range-finding tests and will be advised by Protocol Amendment. Test solutions are prepared to double strength to allow for subsequent dilution by the algal culture.
5.5.	Incubation	The algal cultures are maintained under constant illumination ($\geq$ 4,000 lux) at 23 $\pm$ 2° C and agitated by orbital shaker.
5.6.	Test media renewal	Test media are not renewed.
5.7.	Nutrient medium	The choice of nutrient medium will be given in the Study Details sheet. (See 10. Notes).
5.8.	Control	50 ml algal culture plus 50 ml nutrient medium. A positive control is not employed in this test.

5.9.	Blank	100 ml nutrient medium.
5.10.	Randomization	The flasks are randomly assigned to a position in the culturing apparatus.
5.11.	Compounds of limited water solubility	Where the anticipated EC_{50} value is higher than the limit of water solubility, the desired test series is prepared by dissolving the test substance in a suitable organic solvent (e.g. acetone, DMSO, etc.) to give a series 10^4 times as concentrated as the required test series. To each 100 ml of algal suspension (absorbance value approximately 0.05) is added 10 µl of the various dilutions. In such cases a "solvent" control (at the highest concentration used in the test) is added to the series. Details to be advised by Protocol Amendment.
5.12.	Verification of test concentrations	Details to be advised by Protocol Amendment.

6. Observations

6.1. The algal concentration of each sample is determined at 24, 48 and 72 hours by spectrophotometry. The absorbance of each sample is measured at 665 nm and corrected by substraction of the blank value.

6.2. The actual cell density of the controls is also determined by direct county (haemacytometer) at 0 hours and 72 hours. Three counts are made for each sample.

6.3. Direct counting will be employed for all samples when the absorbance of the test substance would invalidate the spectrophotometer readings.

6.4. Temperature is recorded daily and pH at the start and end of the test.

6.5. Additional recording will be made at 96 hours if requested (details will be given in the Study Details sheet).

7. Results

7.1. The growth curves at each test concentration are constructed by plotting mean absorbance/cell density (logarithmic axix) against time (arithmetic axis).

7.2. The concentration-effect relationship is determined by comparing the areas under the growth curves as calculated by the equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_o}{2} \times (t_2 - t_1) + \ldots +$$

$$\frac{N_{n-1} + N_n - 2N_o}{2} \times (t_n - t_n)$$

Where A = area

N_o = cell concentration/absorbance at start of test

N_1 = cell concentration/absorbance at t_1

N_n = cell concentration/absorbance at t_n

t_1 = time of first measurement after beginning of test

t_n = time of n th measurement after beginning of test

7.3. Percentage inhibition of growth at each test concentration (I_A) is calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c):

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

I_A values are plotted against test concentration and a line fitted by eye. The E_bC_{50} (0-t hours) is read from the graph.

7.4. The average growth rate (maximum) for exponentially growing cultures is also calculated from the straight section of the growth curve by the equation:

$$u = \frac{\ln N_n - \ln N_n}{t_n - t_1}$$

Percentage reductions in growth (rate) and the E_rC_{50} (e.g. 24 h - 48 h) are calculated, as in the previous section, by substituting growth rates for areas under the curve.

8. Reports

8.1. An advance copy of the report will be issued for comment by the Sponsor prior to printing the final report. Unless specified otherwise, six bound copies of the report will be issued. The report will include the following information:

- A description of the test substance;
- A description of the experimental procedures used;
- A summary of all observations and environmental parameters;
- Individual and mean absorbance values at each sampling time;
- The growth curves at each test concentration and control;
- The concentration-effect curve and the calculated E_bC_{50} and E_rC_{50} values;
- The highest concentration tested which gave "no observed effect".

9. Good laboratory practice

9.1. Regulations

The study will be conducted in compliance with the Principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision, Department of Health 1989.

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 (FIFRA) and 792 (TSCA) Federal Register, 29 November 1983 and subsequent amendment Federal Register, 17 August 1989.

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9 Paris 1982.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

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The Study Director will normally seek approval of the Sponsor before any amendment to protocol is made. However, in the event of difficulty in contacting the Sponsor, and for reasons of animal welfare or protection of scientific integrity, the Study Director reserves the right to act without the prior approval of the Sponsor.

9.3. Quality Assurance Unit review

The Quality Assurance Unit will:

(a) Conduct inspections of various phases of the study itself and/or of repetitive procedures relevant to the study design, and

(b) Audit the final report.

9.4. Maintenance of records

Following despatch of the final report, all raw data and other documents generated by HRC during the course of the study, together with a copy of the final report, will be lodged in the Huntingdon Research Centre Ltd. Archive, Huntingdon, United Kingdom.

10. Notes

One of the following nutrient media will be used (to be advised by Protocol Amendment):

2. Nutrient medium:

Solutions

(i)	$NaNO_3$	49.6	g/l
(ii)	K_2HPO_4	3.9	g/l
(iii)	$MgSO_4.7H_2O$	7.5	g/l
(iv)	$CaCl_2.2H_2O$	3.6	g/l
(v)	Ferric-III-citrate	0.3	g/l
(vi)	Trace element solution:		
	H_3BO_3	2.86	g/l
	$MnCl_2.4H_2O$	1.81	g/l
	$ZnSO_4.7H_2O$	0.22	g/l
	$CuSO_4.5H_2O$	0.08	g/l
	$Na_2MoO_4.2H_2O$	0.024	g/l
	$CoCl_2.6H_2O$	0.04	g/l

10 ml of solutions (i) and (ii) 100 ml of solutions (iii), (iv) and (v) 0.4 ml of solutions (vi) 0.46 g $NaHCO_3$ — Made up to 10 litres with deionized water. pH adjusted to 7.0.

[or]

2. Nutrient medium:

Solutions

(i)	NH_4Cl	15.0	g/l
(ii)	$MgCl_2.6H_2O$	12.0	g/l
(iii)	$CaCl_2.2H_2O$	18.9	g/l
(iv)	$MgSO_4.7H_2)$	15.0	g/l

(v)	KH_2PO_4	1.6	g/l
(vi)	$FeCl_3.6H_2O$	0.08	g/l
(vii)	Trace element solution:		
	H_3BO_3	1.850	g/l
	$MnCl_2.4H_2O$	4.150	g/l
	$ZnCl_2$	0.030	g/l
	$CoCl_2.6H_2O$	0.015	g/l
	$CuCl_2.2H_2O$	0.0001	g/l
	$Na_2MoO_4.2H_2O$	0.070	g/l
*(viii)	$Na_2EDTA.2H_2O$	1.00	g/l
	10 ml of solutions (i) to (vi) 1 ml of solutions (vii) and *(viii) 500 mg $NaHCO_3$	Made up to 10 litres with deionized water. The pH of this medium is approximately 8.	

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* Solution (viii) is omitted when testing heavy metals.

ANNEX III

DISCUSSION PROTOCOL

TUNGSTEN AND TUNGSTEN CARBIDE

REPEAT DOSE INHALATION TOXICITY STUDY IN RATS

FOUR WEEKS EXPOSURE WITH POST-EXPOSURE PHASE OF UP TO 16 WEEKS

Sponsor: International Tungsten Industry Association
280 Earls Court Road,
London SW5 9AS

INTRODUCTION

The purpose of this study, to be performed at the Huntingdon Research Centre Limited, Huntingdon, United Kingdom (HRC), is to assess the response in rats to repeat administration, by inhalation, of the test substances for four weeks. The rats will be exposed on five days of each week and some rats from each group will be killed during Week 5.

In this study particular attention will be given to the possible development of lung lesions. Progressive lung lesions, such as fibrosis, are slow in development and therefore some rats will be held for up to 16 weeks post-exposure in order to monitor possible development of such lesions.

The test substances are to be administered by inhalation, a possible route for accidental exposure in man. The rat is the species of choice due to regulatory requirements and the strain is selected on account of the availability of comprehensive background data, relating to clinical and pathological parameters, at HRC laboratories.

A comparative control group, exposed to hard metal powder (tungsten carbide/cobalt mixture), will be included in the study.

The procedures to be used during the course of this study are documented in the relevant HRC Standard Operating Procedures Manuals.

1. Personnel

1.1. Huntingdon Research Centre Limited

1.1.1. Study Director: C.J. Hardy

1.1.2. Study Supervisor: D.W. Coombs

1.1.3. Veterinary Director: D.P. Buist

1.1.4. Principal Pathologist: C. Gopinath

1.1.5. Clinical Pathologist: D. Crook

NOTE: In the temporary absence of the Study Director, the responsibilities of this role will be assumed by G.C. Clark, Head, Department of Inhalation Toxicology.

1.2. Sponsor

1.2.1. Study Monitor: .....

2. Personnel safety

Tungsten, tungsten carbide and hard metal powder (tungsten carbide/cobalt mixture) are dusty materials, the toxicity of which is being investigated. However, HRC standard handling procedures are considered adequate to minimize the risk of exposure of personnel involved in the study.

3. Test system

3.1. Animals: Crl:CD®(SD) BR rats, a Caesarean-derived strain of Sprague-Dawley origin.

3.2. Supplier: Charles River (UK) Limited, Margate, Kent, England.

3.3. Number: 340 (170 males and 170 females).

3.4. Age: Approximately six weeks old on delivery.

3.5. Weight: According to age. Animals will be ordered within a weight range of ± 10 g for each sex.

The rat is selected for use in this study for the following reasons:

- areas of the respiratory tract in the rat are highly sensitive to irritant materials;

- the rat can be used in sufficient numbers to permit statistical evaluation of the study results.

The strain is selected for reason of the availability of comprehensive background data, relating to pathological and clinical parameters, at this laboratory.

3.6. Health inspection: The animals delivered for the study will be inspected by a senior animal technician prior to their allocation to the experimental groups. Any unsuitable animals will be discarded at this time. However, if 10 per cent or more of the animals show signs of ill-health rather than physical damage a Veterinary Officer will be informed. If, in the opinion of the Veterinary Officer, the animals are considered to be not suitable for use in the study, the entire batch of animals will be discarded and a new batch ordered.

3.7. Group allocation: Each animal will be assigned a temporary number, weighed and, when applicable, the required number of animals (including those intended for the reserve group) selected by, for each sex, discarding those animals furthest from the mean bodyweight. The remaining animals will then be randomly assigned to cages, stratified by bodyweight, in such a way that the cage means will be approximately equal. The appropriate numbers of cages will then be allocated to the experimental groups.

3.8. Identification: Each rat will be individually identified by a number tattooed on the ear pinnae.

3.9. Acclimatization: The period of acclimatization will be two weeks approximately.

3.10. Reserve animals: Rats allocated to the reserve group will be used to replace any rat considered unsuitable for experimentation (e.g. low bodyweight gain and/or inappetence, unusual behaviour pattern) or which dies during the acclimatization period. Remaining reserve animals will then be killed.

4. Accommodation and husbandry

4.1. Location of study: Building Y14, room ...

4.2. Cage type and size: Stainless steel mesh on front, back and floor with stainless steel sheet sides: 35 cm wide, 53 cm long and 25 cm high. The cages are suspended on racks. Plastic trays, lined with absorbent paper, will be placed below the cages to collect animal waste.

4.3. Number of animals per cage: Five of the same sex.

4.4. Cage identification: Each cage will bear a coloured label identifying the group (see Appendix 1) and the numbers of the animals contained within it.

4.5. Cleaning of cages: The paper in the trays below the cages will be changed daily. The cages will be changed for clean cages at approximately two-week intervals.

4.6. Room temperature and relative humidity: Will ideally be maintained within the limits 21 ± 3° C and 55 ± 15 per cent. Excursions outside these ranges will be documented as to time and duration of the event. Temperature and relative humidity will be monitored and recorded continuously using a Kent Clearspan recorder.

4.7. Lighting: Will be on a controlled 12-hour cycle; on from 0800-2000 hours, off from 2000-0800 hours.

4.8. Dry Diet: SDS Rat and Mouse No. 1 modified diet. An excess amount of diet will be available to the animals at all times except during inhalation exposures and as specified elewhere in this protocol.

4.9. Water: Tap water, either from individual bottles or from an automatic watering system, will be available to the animals at all times except during inhalation exposures and as listed elsewhere in this protocol. When bottles are used, they will be rinsed and refilled daily.

4.10. Analysis of food: The diet is a closed formula diet suitable for normal health, growth and reproduction of laboratory rats and mice. The standards of production adopted by the manufacturers have been approved by the HRC Quality Assurance Department. Analyses will be made of all batches of diet for most nutrients and for specific substances and micro-organisms likely to be present in feed ingredients or the finished diet and which, if in excess of specified amounts, might have an undesirable effect on the test system. Batches of diet will conform with the standards agreed by the manufacturers and the HRC Quality Assurance Department.

4.11. The water supplied to HRC, by Anglian Water, is potable water for human consumption. Anglian Water takes its guidelines on water quality from the EEC directive relating to water for human consumption, viz: Council Directive 80/778/EEC.

Results of routine physical and chemical examination of drinking water at source as conducted, usually weekly by the supplier, are made available to HRC as quarterly summaries.

These results include levels of:

Nitrites	Potassium	Chloride
Nitrates	Silicon	Iron
Calcium	Arsenic	Selenium
Magnesium	Barium	Silver
Sodium	Antimony	Phosphorus

as well as concentrations of pesticides, related products, polycyclic aromatic hydrocarbons, haloforms, chlorophenols and polychlorinated biphenyls.

5. Test substance

5.1. Test substances:

5.1.1. (a) Chemical name: Tungsten

(b) Common name:

(c) Presentation:

(d) Received from:

(e) On:

(f) Batch No.:

(g) Purity:

(h) Stability:

(i) Expiry date (for batch supplied):

5.1.2. (a) Chemical name: Tungsten carbide

(b) Common name:

(c) Presentation:

(d) Received from:

(e) On:

(f) Batch No.:

(g) Purity:

(h) Stability:

(i) Expiry date (for batch supplied):

5.1.3. (a) Chemical name: Tungsten carbide/cobalt mixture

(b) Common name: Hard metal powder

(c) Presentation:

(d) Received from:

(e) On:

(f) Batch No.:

(g) Purity:

(h) Stability:

(i) Expiry date (for batch supplied):

5.2. Storage: Room temperature and humidity, unless otherwise specified.

5.3. Disposal: Unused test substance remaining at the end of the study will be returned to the Sponsor or will be disposed of by the Huntingdon Research Centre Limited. Any costs incurred in disposal or return of the test substance will be the responsibility of the Sponsor and will be advised separately. Any such costs are not included in the study cost shown on the quotation.

6. Route of administration

The inhalation route is selected since this is a possible route of accidental exposure in man.

7. Administration

7.1. Exposure levels: The exposure levels will be selected in consultation with the Sponsor and taking account of all available data, including the results of preliminary studies.

7.2. Groups and exposure levels: The allocation of animals to the experimental groups, group numbers and provisional exposure levels are shown in Appendix 1.

7.3. Exposure: The test aerosols will be administered by inhalation as described below.

7.3.1. Exposure duration: A single six-hour exposure on five days (Monday to Friday) of each week for four weeks.

The rats in Group 2 (Comparative control) will be exposed to "hard metal powder", a mixture of tungsten carbide and cobalt.

7.3.2. Exposure system. Rats will be exposed in whole-body exposure chambers constructed from stainless steel and glass. The internal volume of the chamber is approximately 675 litres.

Air will be introduced into the exposure chamber at a total rate of 150 litres per minute. This will be made up from:

Atmosphere generator: * litres per minute

Diluent introduced into the elutriation column: * litres per minute

The exposure chamber will be maintained 10 mm H_2O below ambient pressure by an extraction unit connected to the exhaust plenum.

Rats will be housed singly in stainless steel mesh compartments.

7.3.3. Generation of test atmosphere: The test atmospheres will be generated using a Wright dust feed mechanism or suitable alternative. Details of the generation system will be provided in the final protocol. a/

8. Chamber conditions

8.1. Concentration: At least three air samples will be taken from each exposure chamber (except control) during each exposure, to determine the concentration of test substance in air. Air samples will be taken from the level(s) used to expose the rats. Additional samples will be taken as considered necessary.

An appropriate sample collection procedure will be developed during preliminary studies and details will be provided in the final protocol.

8.2. Particle size: A single air sample will be taken from each exposure chamber (except control), during each exposure to determine the aerosol particle size.

Samples will be taken using a Marple (Model 296) Personal Cascade Impactor. A sampling rate of 2 litres per minute will be used. Aerosol will be collected on stainless steel substrate.

8.3. Method of analysis of the test substance in air: Gravimetric.

8.4. Airflow: The airflow through the chamber will be 150 litres/minute and will be monitored continuously using tapered tube rotametres. The airflow will be recorded at 30-minute intervals.

8.5. Chamber temperature and relative humidity: Chamber temperature and relative humidity will be recorded continuously using a wet/dry bulb hygrometer and recorded at 60-minute intervals.

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a/ The choice of device will be made during preliminary experimentation and will be determined by the physical properties of the test substance.

9. Clinical observations

Dated and signed records of all activities relating to the day-by-day running and maintenance of the study, as well as to the group observations and examinations as listed in this protocol, will be recorded in the study Day Book. Dated and signed records of appearance, change and disappearance of clinical signs will be maintained on clinical history sheets for individual animals.

9.1. Daily checks: Throughout the study, all cages will be checked in the morning and again in the afternoon for dead or moribund animals.

9.2. Mortality and morbidity: Any animal found dead or dying during the acclimatization period will be discarded and replaced by an animal from the reserve group. Any animal found dead or dying at any other time will be examined macroscopically and a full spectrum of tissues preserved (section 11.5.). Where possible, dead and moribund animals will be subjected to macroscopic examination on the day of death. Otherwise, the carcass will be placed inside a polythene bag and kept in a refrigerator (4° C) until examination on the following day.

9.3. Clinical signs: Signs of ill-health, together with any behavioural change or response to treatment, will be recorded. Individual animal records will be maintained on the basis of:

- any observation, considered to be of possible importance, made at any time during the study;

- a careful external examination made at weekly intervals when special attention will be given to the detection of audible respiratory noise.

During exposure, signs will be recorded as a group response where all visible rats appear to be reacting similarly to the test substance.

9.4. Bodyweight: The weight of each rat will be recorded weekly beginning one week before the start of dosing. During the dosing period, the bodyweights will be recorded before dosing on the day.

9.5. Food consumption: The amount of food consumed by each cage of rats will be recorded on a weekly basis beginning one week before the start of dosing.

9.6. Water consumption: Water consumption may be introduced if an effect on water consumption is suspected from gross observation of the water loss from the drinking bottles.

NOTE: This may involve an additional cost to the Sponsor.

10. Laboratory investigations

10.1. Sample interval: Blood samples (for analysis as detailed below) will be collected from five male and five female rats in each group on one occasion during Week 4 of dosing.

If necessary, examination will be extended to the rats killed six or sixteen weeks post-exposure at additional cost to the Sponsor.

10.2. Blood collection: Samples of venous blood will be withdrawn from the orbital sinus while the rats are held under light ether anaesthesia. Food will be withheld from all animals overnight prior to sampling.

10.3. Anticoagulants: The blood samples collected will be divided into tubes containing the following anticoagulants:

EDTA - for haematological investigations.

Citrate - for clotting test.

Heparin - for blood chemistry investigations.

10.4. Urine collection: If an effect on water or electrolyte balance is suspected, urine collection may be undertaken at additional cost to the Sponsor. Details of the procedures to be used would be issued in a protocol amendment.

10.5. Repeat investigations: At the discretion of the Study Director, results found to be abnormal may be checked by obtaining further samples of blood and repeating the investigation. These values will be recorded as a footnote in the relevant appendix of the report.

10.6. Haematology: The following estimations will be performed with an Ortho ELT-1500 haematology analyser, using standard Ortho methodology:

Packed cell volume (PCV)	per cent
Haemoglobin (Hb)	g/dl
Red cell count (RBC)	x10 mm^6/mm
Mean corpuscular haemoglobin concentration (MCHC) - by calculation, Hb (g/dl) x 100 / PCV (%)	per cent
Mean corpuscular volume (MCV) - by calculation, PCV (%) x 10 / RBC ($x10^6/mm^3$)	fl
Total white cell count (WBC total)	$x10^3/mm^3$
Platelet count (Plts)	$x10^3/mm^3$

The following will be performed using the appropriate methodology, as described below:

Differential count (Diff) - standard microscopy of blood smear, stained with modified Wright's stain, counting 100 cells:

Neutrophils	(N)	
Lymphocytes	(L)	
Eosinophils	(E)	$x10^3/mm^3$
Basophils	(B)	
Monocytes	(M)	

Cell morphology - if abnormal cells are observed when examining any stained slide, their presence will be recorded.

Results for polychromasia, hypochromasia, anisocytosis and rouleaux will be reported on a scale 1 (slight) to 4 (gross).

Thrombotest (TT) - method of Owren, P.A., Lancet, 1959, ii, 754	s
Reticulocyte count (retic) - Method of Dacie, J.V. and Lewis, S.M. (Practical Haematology, 1966. 3rd edit. p. 28)	per cent (of red cells)

10.7. Biochemistry: The following parameters will be analysed with a Roche Cobas centrifugal analyser, using the appropriate BCL test kit:

Creatinine phosphokinase (CPK), also known as "creatinine kinase" - reaction temperature 30° C	mU/ml

The following parameters will be analysed with a Hitachi 737 clinical chemistry analyser, using standard Hitachi 737 methodology:

Total protein	g/dl
Albumin (Alb)	g/dl
Globulin (Glob) - by subtraction, total protein (g/dl) minus albumin (g/dl)	g/dl
Urea nitrogen (Urea Nitr)	mg/dl
Alkaline phosphatase (AP) - reaction temperature 30° C	mU/ml
Total bilirubin	mg/dl
Creatinine	mg/dl
Sodium (Na)	mEq/l
Potassium (K)	mEq/l
Calcium (Ca)	mEq/l

Inorganic phosphorus (P)	mEq/l
Chloride (Cl)	mEq/l
Cholesterol (Chol)	mg/dl
Glucose - hexokinase mediated	mg/dl
Glutamic-pyruvic transaminase (GPT), also known as "alanine aminotransferase" - reaction temperature 30° C	mU/ml
Glutamic-oxaloacetic transaminase (GOT), also known as "aspartate aminotransferase" - reaction temperature 30° C	mU/ml
Gamma-Glutamyltransferase (γGT), - reaction temperature 30° C	mU/ml

11. Terminal studies

11.1. Time of terminal studies:

Following four weeks of dosing, five male and five female rats from each group will be killed.

Six weeks after the end of dosing, five male and five female rats from each group will be killed.

Sixteen weeks after the end of dosing the remaining 10 male and 10 female rats in each group will be killed.

When sacrifice of the animals takes place over more than one day, approximately equal numbers of rats from all groups will be killed on each day (assuming numbers are not markedly altered by early deaths).

11.2. Method of sacrifice: The rats will be anaesthetised by an intraperitoneal injection of pentobarbitone sodium and exsanguinated from the brachial arteries.

11.3. Macroscopic examination: A detailed macroscopic examination of each animal will be performed.

11.4. Organ weights: The organs marked "X" in Table 1 will be weighed.

11.5. Tissue preservation: From each animal, samples or the whole of the tissues listed in Table 1 will be preserved in 10 per cent neutral buffered formalin; eyes which will be preserved in Davidson's fixative.

11.6. Histopathology: The tissues will be embedded in paraffin wax and sections approximately 4 micrometres thick will be cut, processed and stained with haematoxylin and eosin for examination by light microscopy. Other stains might be used to aid the diagnosis of lesions.

Histopathological examination will be performed as follows:

Rats killed at the end of the exposure period -

Groups 1, 2, 5 and 8

Lungs, trachea, larynx, nasal turbinates, spleen, kidneys, liver, sternum for bone and marrow, tracheobronchial lymph nodes.

Groups 3, 4, 6 and 7

Lungs and tracheobronchial lymph nodes.

Rats killed six or sixteen weeks post exposure -

All groups:

Lungs, and tracheobronchial lymph nodes.

If changes are seen in tissues other than the lungs and tracheobronchial lymph nodes in Groups 5 or 8 at the end of exposure, it will be necessary to examine the affected organs for other groups at other kill times. This will involve additional cost to the Sponsor.

12. Statistical analysis

Analysis of variance followed by Student's "t" and Williams' tests are most commonly employed to assess the significance of intergroup differences when data suggest evidence of a response to the administration of the test substance (see Appendix 2). However, other statistical methods may be employed at the discretion of the Study Director.

13. Reports

13.1. Interim reports: The Sponsor will be informed as soon as possible of any unexpected toxicity and/or response to administration of the test substance.

13.2. Final report: Five bound copies and one unbound copy of the report will be provided. The data presented in the report will include but not be limited to the following:

- Strain, age, weight and source of animals used;
- Description of the method of allocation of animals to groups;
- Environmental conditions;
- Description of system and procedures for inhalation exposure;
- Results of the chamber concentration and particle size analyses;

- Clinical signs;
- Individual animal and group mean bodyweights;
- Food consumption data by cage and group;
- Results of blood tests;
- Individual macroscopic examination reports;
- Organ weight data;
- Individual histopathological findings;
- Description of all statistical procedures used.

14. Archives

All raw data collected and a sample from each batch of test substance supplied during the study will be stored in HRC's purpose-built archive facility. Study files will be microfilmed, with one copy stored in a separate location from the archive. Raw data will be stored for as long as the quality permits evaluation. Wet tissues will be stored no longer than 10 years from the date of submission of the study report. The Sponsor will be approached prior to disposal of any raw data.

15. Good Laboratory Practice and Quality Assurance

This study will be conducted in compliance with the principles of Good Laboratory Practice as set forth in:

United States Environmental Protection Agency, (TOSCA) Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent Amendment, Federal Register, 17 August 1989.

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision. Department of Health, 1989.

The HRC Quality Assurance Department will review the protocol for GLP completeness, maintain a record of the study on its master schedule, inspect the study procedures and audit the final report for accuracy of presentation and collation of the raw data.

16. Records to be maintained

These are listed in Appendix 3.

17. Time plan

This is shown in Appendix 4.

18. Amendments

Amendments to this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such change will be honoured by HRC. However, it then becomes the responsibility of the Sponsor to follow such verbal authorization with a written verification. All protocol modifications will be issued and signed by the Study Director and Sponsor.

Table 1

Allocation of tissues at post-mortem

Tissues to be preserved	Organ weighed
Adrenals	
Alimentary tract	
Oesophagus	
Stomach (glandular and non glandular)	
Duodenum	
Jejenum	
Ileum	
Caecum	
Colon	
Rectum	
Animal identification mark	
Aorta	
Brain	
Eyes	
Femur with joint (for bone and marrow in situ)	
Gross abnormalities	
Heart	
Kidneys	X
Larynx	
Liver	X
Lungs (all lobes and mainstem bronchi)	X
Lymph nodes (cervical, mesenteric and tracheobronchial)	
Mammary gland	
Nasal passages (head for rostral and caudal nasal cavities)	
Optic nerve	
Ovaries	
Oviduct	
Pancreas	
Pharynx	
Pituitary	
Prostate	

Table 1 (continued)

Tissues to be preserved	Organ weighed
Salivary gland	
Sciatic nerve	
Seminal vesicles	
Skeletal muscle (thigh)	
Skin	
Spinal column	
Spinal cord (cervical, thoracic and lumbar)	
Spleen	X
Sternum	
Testes (with epididymides)	
Thymus (where present)	
Thyroids (with parathyroids)	
Tongue	
Trachea (including bifurcation)	
Ureter	
Urinary bladder	
Uterus (corpus and cervix)	
Vagina	

X All rats from all groups.

The nasal cavity will be flushed with fixative prior to immersion.

The lungs will be infused with fixative prior to immersion.

The lymph nodes will be identified separately.

The remaining head will be retained for paranasal sinuses, oral cavity, nasopharynx, middle ear, teeth, eyelids, lachrymal gland, Harderian gland and Zymbal's gland.

Appendix 1

Experimental groups, animal numbers and exposure concentrations and schedule

Group		Rat numbers		Test substance concentration in air*
		Male	Female	(mg/m^3)
1	(Control)	1- 20	161-180	0
2	(Hard metal powder)	21- 40	181-200	100
3	(Low dose Tungsten)	41- 60	201-220	10
4	(Inter. dose Tunsten)	61- 80	221-240	100
5	(High dose Tungsten)	81-100	241-260	500
6	(Low dose Tungsten carbide)	101-120	261-280	10
7	(Inter. dose Tungsten carbide)	121-140	281-300	100
8	(High dose Tungsten carbide)	141-160	301-320	500
	Reserve	321-330	331-340	

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* Subject to review.

Rats will be exposed for six hours a day for five days per week for four weeks.

The following colour codes will be used to identify cage labels and doumentation associated with each group:

Group	Colour code
1	White
2	White with black edges
3	Yellow
4	Green
5	Red

Appendix 2

Statistical analyses

All statistical analyses will be carried out separately for males and females.

Bodyweight data will be analysed using weight gains.

The following sequence of statistical tests will be used:

(i) If the data consist predominantly of one particular value (relative frequency of the mode exceeds 75 per cent), the proportion of animals with values different from the mode will be analysed by appropriate methods. Otherwise:

(ii) Bartlett's test (1) will be applied to test for heterogeneity of variance between treatments. Where significant (at the 1 per cent level) heterogeneity is found, a logarithmic transformation will be tried to see if a more stable variance structure can be obtained.

(iii) If no significant heterogeneity is detected (or if a satisfactory transformation is found), a one-way analysis of variance will be carried out. If significant heterogeneity of variance is present, and cannot be removed by a transformation, the Kruskal-Wallis analysis of ranks (2) will be used.

(iv) Except for pre-dose data, analyses of variance will be followed by Student's "t" test and Williams' test (3) for a dose-related response, although only Williams' may be reported. The Kruskal-Wallis analyses are followed by the non-parametric equivalents of the "t" test and Williams' test (Shirley's test (4)).

Where appropriate, analysis of covariance is used in place of analysis of variance in the above sequence. For most parameters, the appropriate covariate is the same parameter at pre-dose. For organ weight data, the final bodyweight is used as a covariate in an attempt to allow for differences in bodyweight which might influence the organ weights.

References

1. Bartlett, M.S., (1937), Proc. Roy. Soc. Series A., 160: 268-282.

2. Kruskal, W.J. and Wallis, W.A., (1952/3). J. Amer. Statist. Ass., 47: 583-621 and 48: 907-912.

3. Williams, D.A., (1971/2), Biometrics, 27: 103-117 and 28: 519-531.

4. Shirley, E., (1977), Biometrics, 33: 386-389.

Appendix 3

Records to be maintained

1. Protocol and protocol agenda
2. Study schedule
3. Pre-initiation data
4. Technical personnel list with signatures
5. Source, purchase order and delivery note of animals
6. Sex verification
7. Randomization data
8. Animal identification
9. Diet batch number and analysis
10. Drinking water analyses
11. Room humidity and temperature
12. Test article data
13. Test article administration
14. General procedures
15. Clinical signs
16. Bodyweight data
17. Food consumption data
18. Clinical laboratory data
19. Date and type of termination for each animal in the study
20. Necropsy findings and organ-weight data
21. Histopathological findings
22. Quality assurance
23. Miscellaneous study data

Appendix 4

Time plan

Date ( )	Week	Activity
	-2	Animals arrive Veterinary inspection Ophthalmoscopy Allocation to groups Kill any surplus animals
	-1	Begin routine measurement of bodyweight and food consumption
	1	Begin exposure Kill reserve animals
	4	Haematology, biochemistry (5/sex/group) Last exposure
	5	Post-mortem 1 (5/sex/group)
	10	Post-mortem 2 (5/sex/group)
	20	Post-mortem 3 (10/sex/group)